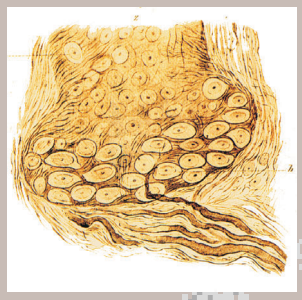
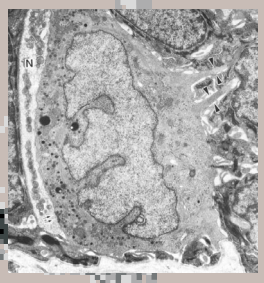
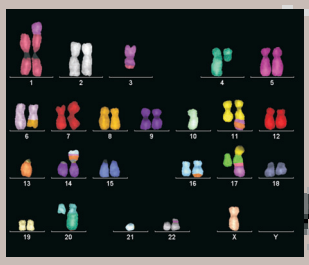
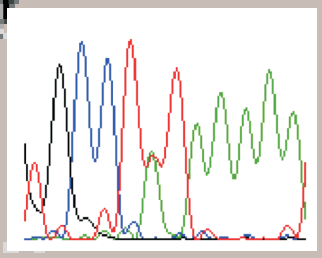
Study of genomic aberrations and gene expression profiling in Merkel cell carcinoma

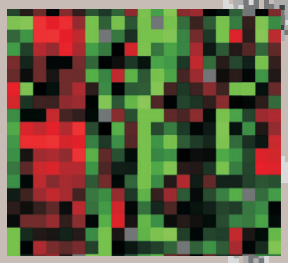
F. Sigmund Merkel, 1875











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"Study of genomic aberrations and gene expression profiling in Merkel cell carcinoma"

this thesis is submitted as fulfillment of the requirements for the degree of
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Dedicated to my parents and Geert

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PART 1

Introduction

1.1 The genetic basis of cancer

Over the past 30 years, considerable information has been gathered on regulation of cell growth and proliferation which led to the discovery of many cancer-associated genes. This complex body of knowledge revealed that cancer is the result of many dynamic changes in the genome influencing a variety of cellular mechanisms such as cell cycle control, apoptosis, angiogenesis, cell adhesion and motility (Figure 1-1) (Hanahan and Weinberg, 2000).

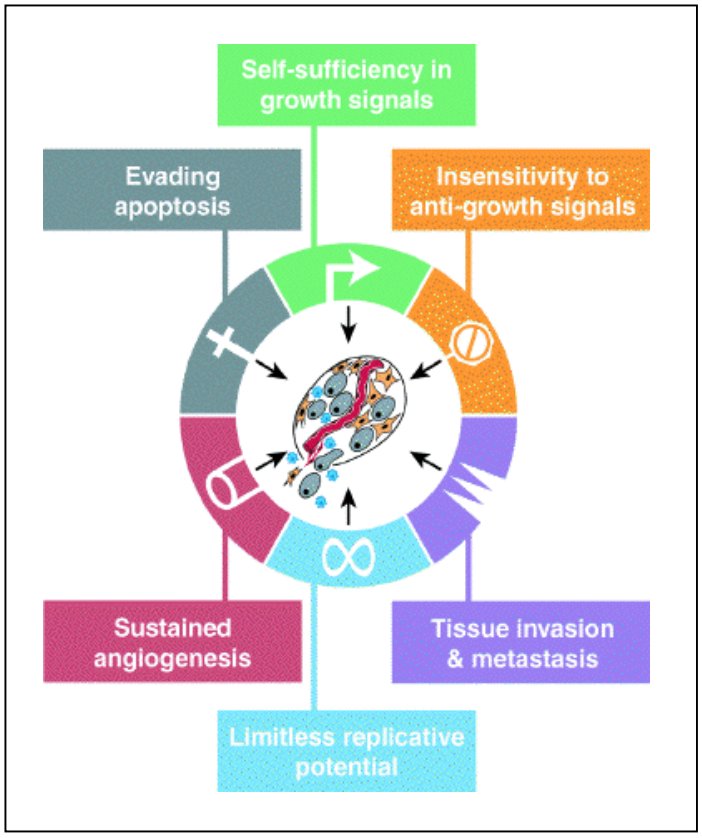


Figure 1-1: Acquired capabilities of cancer. It is suggested that the defects leading to cancer are a manifestation of six alterations in cell physiology that collectively dictate malignant cell growth: (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals, (3) evading apoptosis, (4) limitless replication potential, (5) sustained angiogenesis and (6) acquiring invasive and metastatic properties.

(Reproduced from Hanahan and Weinberg, Cell, 100, 57-70, 2000.)

A short overview of the key discoveries which have led to our current view and understanding of cancer development is given below.

In 1890, the German biologist David von Hansemann was the first to observe several spindle bodies and other mitotic aberrations in tumor cells (von Hansemann, 1890). More then twenty years later, Theodor Boveri proposed that chromosome defects in a given cell were the underlying cause of cancer (Boveri, 1914). Boveri's idea lacked experimental support, which was not provided until the discovery by Peter Nowell and David Hungerfold in 1960 (Nowell and Hungerfold, 1960). They described the Philadelphia chromosome in chronic myelogenous leukemia (CML) cells. Following the introduction of chromosome banding, this Philadelphia chromosome was shown to result from a reciprocal translocation between chromosomes 9 and 22 (Rowley, 1973). With further improvement of cytogenetic methods many other non-random chromosomal rearrangements were identified in leukemic disorders as well as in solid tumors (Mitelman, 2000). The frequent association between certain chromosome rearrangements and specific tumor types was strongly suggestive for a causal role of these genetic alterations in the development of cancer. Moreover, certain chromosome rearrangements were strong prognostic indicators and were of diagnostic relevance in these cases.

The discovery of cellular proto-oncogenes (Stehelin *et al.*, 1976) paved the way to a mechanistic understanding of how these translocations lead to cancer. In a first type of rearrangements, translocations can lead to an aberrant expression of certain proto-oncogenes. This was illustrated by the t(8;14) (q24;q32) translocation in Burkitt's lymphoma which resulted in a translocation of the *MYC* oncogene into the 5' region of the immunoglobulin heavy chain (*IGH*) gene. The translocation juxtaposed the *IGH* promoter region with the *MYC* coding sequences, resulting in abnormal timing and levels of *MYC* expression and leading to a proliferative advantage of the transformed cell (Taub *et al.*, 1982; Adams *et al.*, 1985; Rowley, 2001). A second type of genetic aberration was the formation of tumor-specific fusion genes. This was examplified by the above mentioned t(9;22) translocation in CML which resulted in fusion of the *ABL* and *BCR* genes (Shtivelman *et al.*, 1985; Rowley, 2001). The resulting chimeric BCR/ABL protein showed an increased tyrosine kinase activity leading to a deregulation of cell cycle control and apoptosis, through activation of the AKT oncoprotein (Skorski *et al.*, 1997). Other mechanisms which lead to inappropriate activation of proto-oncogenes are point mutations, as examplified by these occurring in members of the *RAS*-family (Tabin *et al.*, 1982), and gene amplifications which manifest as homogeneously staining regions or double-minute chromosomes in tumors. One of the first oncogenes identified in this way were *MYCN*, in

neuroblastomas (Schwab *et al.*, 1983) and *MYCL*, in small cell lung carcinomas. Although they are a less frequent cause, viral infections can also play a role in tumor formation. This is the case in several cervical cancers and some hepatomas (Weinberg, 1994). From the above data it is clear that proto-oncogenes can be activated by different mechanisms leading to a disruption of their control of normal cell growth. However, increased activity of a single proto-oncogene is generally not sufficient to induce malignant transformation of a normal cell leading to the concept that multiple proto-oncogenes work together to transform cells (Weinberg, 1985). Moreover, proto-oncogenes work in concert with a wide variety of other genetic changes to eventually produce the malignant phenotype. One group of genes interacting with the proto-oncogenes are tumor suppressor genes.

In contrast to the dominantly acting proto-oncogenes, tumor suppressor genes are genes whose loss or inactivation contributes to malignant transformation. The existence of tumor suppressor genes was evidenced from studies of hereditary cancers which showed a clear pattern of inheritance and a tendency for earlier age of onset as compared to sporadic tumors. Based on population incidence curves, Alfred Knudson proposed that two genetic events or 'hits' were sufficient to develop retinoblastoma (Knudson, 1971, 2001). In inherited cases, one of these hits was transmitted through the germ-line while in the sporadic form of the disease both hits were acquired at the somatic level. Studies of retinoblastoma patients with cytogenetically constitutional deletions and loss of heterozygosity (LOH) showed consistent involvement of the chromosomal region 13q14 (Cavenee, 1983) finally leading to cloning of the first tumor suppressor gene, the retinoblastoma gene (*RB1)* (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). Further studies showed that the *RB1* gene played a central role in the cell cycle control and cell differentiation pathways which were disrupted in most of the cancer types investigated (Weinberg, 1995). The discovery of the *RB1* gene provided a molecular basis for Knudson's 'two-hit' hypothesis as the proposed two hits affected both alleles of a single gene, hence the concept of tumor suppressor gene inactivation as a basis of tumor predisposition was born. Today, Knudson's model still holds true for most tumor suppressor genes (Macleod, 2000). However, the notion that loss of a single allele or haploinsufficiency can be sufficient to promote tumor formation or progression was recently reported for tumor suppressor genes involved in familial cancer syndromes (i.e *APC* and *PTEN*) (Di Cristofano *et al.*, 1998; Kwabi-Addo *et al.*, 2001; Yan *et al.*, 2002) pointing at another mechanism of tumor suppressor gene inactivation. Tumor suppressor

genes which directly intervene in prevention of tumor formation have also been called 'gatekeepers' and include the *RB1* (retinoblastoma) (Figure 1-2), *NF1* (Neurofibromatosis type I), *VHL* (von Hippel-Lindau) and *APC* (Adenomatous polyposis coli) genes (Kinzler and Vogelstein, 1997; Macleod, 2000). Their loss of function is rate-limiting for tumor initiation and restoring 'gatekeeper' function in tumor cells suppresses neoplasia. By contrast, 'caretaker' tumor suppressor genes are indirectly associated with tumor initiation. Inactivation of a caretaker leads to genetic instability and accumulation of additional mutations. Many caretakers are DNA repair genes such as the *MSH2* and *MLH1* genes involved in Hereditary Nonpolyposis Colorectal Cancer (HNPCC) or the ATM protein kinase. Loss of the caretaker function predisposes to cancer by increase of the DNA mutation rate and increased chances for loss of function mutations in gatekeeper genes. As a consequence, restoring the function of a caretaker gene would not stop tumor growth if mutation of one or more gatekeepers had already taken place. The definitions of gatekeepers and caretakers are flawed when applied to *TP53*. As an inducer of apoptosis, *TP53* appears to act as a gatekeeper but in its role as "the guardian of the genome", *TP53* can clearly be defined as a caretaker whose major function is to prevent genomic instability. Finally, some tumor suppressor genes function as 'landscapers'. The 'landscaper' phenomenon was first described in patients with juvenile polyposis syndrome (JPS) wherein the initiating lesions occurred in the stromal cells surrounding the tumor and not in the tumor cells themselves (Kinzler and Vogelstein, 1998). The landscaper tumor suppressor genes act by modulating the microenvironment in which tumor cells preferentially will grow by direct/indirect regulation of extracellular matrix proteins, cell surface markers, adhesion proteins or secreted growth/survival factors. In other words, loss of function of a 'landscaper' causes the microenvironment to either function or grow aberrantly, promoting the neoplastic conversion of adjacent epithelia. Since we know that germline mutations in *PTEN* and *DPC4* have both been associated with JPS it would be interesting to determine whether the stromal or epithelial compartment of hamartomatous lesions show *PTEN* or *DPC*4 mutations.

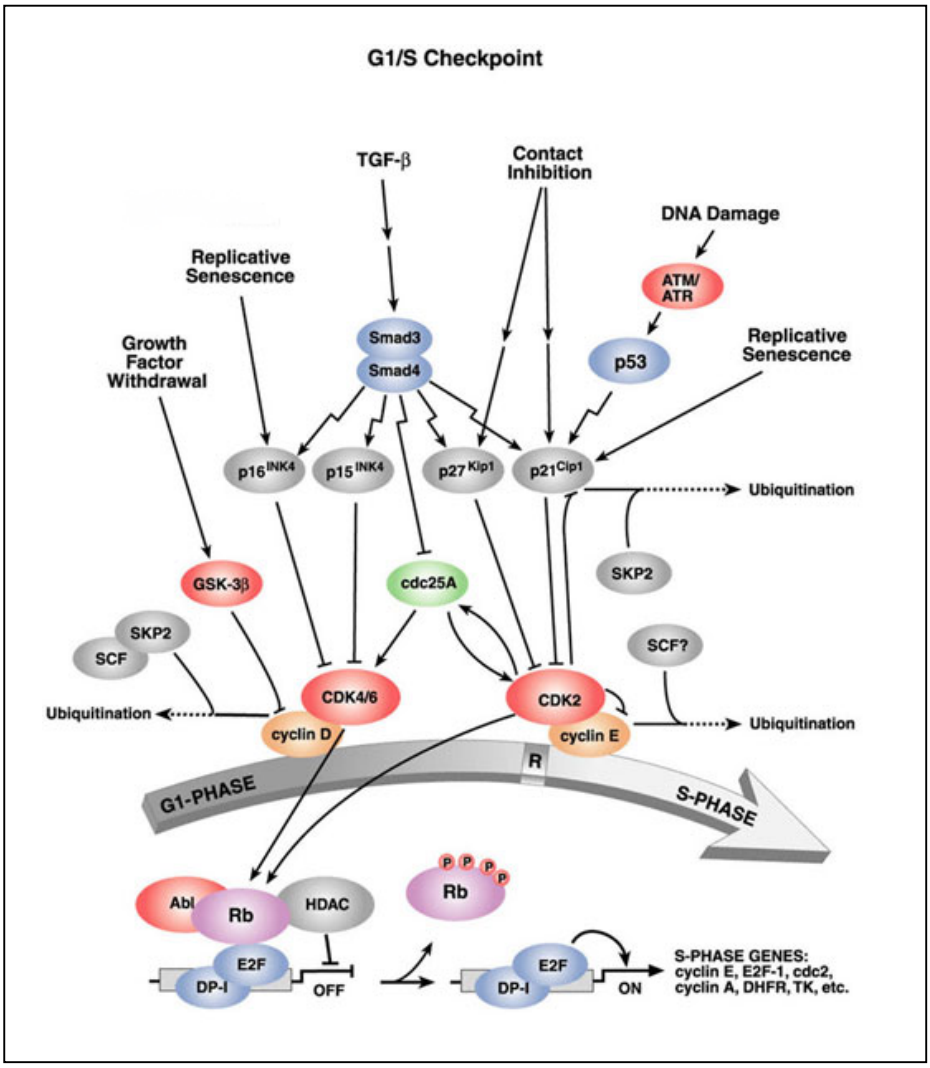


Figure 1-2: Central role of the Retinoblastoma (*Rb*) tumor suppressor gene in the G1 to S checkpoint control of the cell cycle.

(Detailed information is available at http://www.cellsignal.com/retail/reference/pathway/G1S.asp)

From the above presented data it is clear that cancer does not arise by a single mutation in a proto-oncogene or by loss of both alleles of a tumor suppressor gene. It is now widely accepted that cancer occurs as a consequence of several mutations in multiple genes. This 'multi-step' model is well illustrated by studies of colorectal cancer. In this tumor at least seven independent genetic defects appear to be required to transform a normal colon epithelial cell to a metastasizing carcinoma (Kinzler and Vogelstein, 1996). It has been argued that an underlying genetic instability is absolutely required for the generation of multiple mutations that underlie cancer. There is now evidence that most cancers may be genetically unstable and that instability can occur in two different forms. The so-called 'mutator' phenotype, first observed in colorectal cancer, is caused by mutations in one or several mismatch repair genes. In patients with HNPCC and some sporadic colorectal cancers, nucleotide changes occur at high frequency in microsatellite repeats and other repetitive tracts (Kinzler and Vogelstein, 1996). This genetic instability is termed microsatellite instability or MIN (Thibodeau *et al.*, 1993). MIN tumors are, in general, diploid and do not show increased rates of chromosome losses and gains. By contrast, almost 85% of colorectal cancers are highly aneuploid. This nearly always results in karyotypic variability from cell to cell. This form of genetic instability, termed chromosomal instability or CIN (Lengauer *et al.*, 1997, 1998), has also been observed in other solid tumors, including breast, prostate, oropharynx and lung tumors. Unlike MIN tumors, CIN colon cancers generally do not have instability at the nucleotide level. The molecular mechanism of CIN remains a mysterious and somewhat controversial issue in cancer research. Yeast studies have identified more than 100 genes, which gave raise to CIN when mutated in yeast cells (Spencer *et al.*, 1990). Many of these genes have several homologues in humans and are involved in chromosome metabolism, spindle assembly and dynamics, cell cycle regulation and checkpoint control (Lengauer *et al.*, 1998). One gene proposed as a potential 'master' initiator of CIN is the *APC* tumor suppressor gene (Fodde *et al.*, 2001; Kaplan *et al.*, 2001). Another class of CIN candidates are those genes involved in the mitotic-spindle checkpoint and sister-chromatid separation pathways (Amon, 1999; Jallepalli and Lengauer, 2001). Studies showed that defects in such pathways can lead to the types of chromosome loss observed in CIN cancers (Cahill *et al.*, 1998; Michel *et al.*, 2001). Overall, the above mentioned data show that, at least in colon, MIN and CIN are essentially equivalent mechanisms for acquiring the genetic alterations that are needed for tumorigenesis. Whereas CIN seems to be the predominant pathway in most sporadically

occurring solid tumors, hereditary mutations that result in MIN seem to bypass the requirement for CIN during colorectal tumorigenesis.

An essential step in tumor progression is the acquisition of limitless replicative potential. In contrast to normal human cells, which proliferate for a limited period of time before entering senescence (irreversible growth arrest), tumor cells escape replicative senescence, proliferate indefinitely and become immortal. Interestingly, tumor cells have acquired the ability to maintain their chromosomal ends, called telomeres, at a stable length. The enzyme responsible for adding telomeric repeats to the chromosome ends, telomerase, was shown to be activated in 90% of tumors. Re-activation of telomerase activity is thereby an essential mechanism for ongoing proliferation and malignancy of tumor cells. Telomerase is consequently not only a target for cancer diagnosis but also for the development of novel anti-cancer therapeutic agents (Shay *et al.*, 2001; Stewart and Weinberg, 2002).

At a certain time during the development of most human cancer types, cancer cells will escape from the primary tumor mass, invade adjacent tissue and travel through blood vessels or lymphatic system to distant sites of the body where they can extravasate and form new tumor colonies or metastases. The process of invasion and metastasis is the result of interactions in a microsystem involving cancer cells, host cells and extracellular matrix as the basic elements and in which genes coding for extracellular proteases, integrins and cell-cell adhesion molecules play a key role (Mareel *et al.*, 1995). Analogous to the regulation of oncogenes and tumor suppressor genes during tumor development, the regulation of invasion-promoter and invasion-suppressor complexes plays a crucial role in the progression to the invasive phenotype. For example, experimental studies have convincingly shown that the E-cadherin/catenin complex exerts an invasion-suppressor function (Vleminckx *et al.*, 1991) and mediates an important role in cell-cell adhesion and signal transduction (Vermeulen *et al.*, 1996). Downregulation of any of the elements in this complex has been observed in nearly all forms of cancer to be accompanied with invasion and metastasis indicating that its functional elimination is a key step in the acquisition of metastatic properties. Invasion and metastasis are associated with the highest degree of malignancy rendering the disease most of the time incurable.

1.2 Merkel cell carcinoma

Merkel cell carcinoma (MCC) is a rare aggressive neuroendocrine skin tumor first described by Toker in 1972. The disease affects mostly elderly people and occurs predominantly on the sun-exposed areas of the skin. MCC is thought to be derived from Merkel cells, although formal proof for this assumption is lacking. Merkel cells are mainly located in the basal epidermis and have properties of both epithelial and neuroendocrine cells. Their structure, function and origin will be discussed in more detail below.

1.2.1 Merkel cells: distribution, structure and function

The German anatomist and histopathologist Friedrich Sigmund Merkel showed a great interest in the possible existence of receptor cells for touch sense in the skin. After perfecting staining and fixation techniques, Merkel discovered peculiar cells associated with nerve fibers in mammalian and avian skin, and called these cells "Tastzellen" or "touch-cells" (Merkel, 1875, 1878). These cells are now called Merkel cells. He postulated that these cells functioned as receptors for mechanical stimuli in all animals, from amphibians to man.

It is now known that Merkel cells are widely distributed in the skin and oral mucosa of every vertebrate class. In addition, they can be found in the outer root sheet of all sinus hair follicles and most vellus hair follicles. Merkel cells occur also as serotonergic basal cells in the basal portion of taste buds in some lower vertebrates such as the frog. There they are responsible for the initiation of taste bud morphogenesis (Toyoshima *et al.*, 1999) and for the maintenance of the morphological integrity of taste buds (Hamasaki *et al.*, 1998).

Mammalian Merkel cells typically appear as large, oval, clear cells in or near the basal layer of the epidermis. By contrast, in birds they are localized in the dermal connective tissue. Merkel cells are often difficult to visualize by light microscopy and may be confused with other clear cells such as melanocytes and Langerhans cells. With the introduction of electron microscopy, Merkel cells were easier identified and their structure could be studied in more detail (Cauna, 1962). Ultrastructurally, Merkel cells are characterized by the following features: 1) numerous dense cored granules of 80-100 nm in

diameter, which show a tendency to accumulate at the attaching site of the nerve terminal; 2) Golgi-complexes in the cytoplasm opposite the major aggregation of dense cored granules; 3) many microvilli extending randomly from the cell surface; 4) desmosomal attachments to neighbouring keratinocytes; 5) highly pleomorphic nucleus with several deep invaginations; 6) frequent contact with nerve terminals; and 7) occasional appearance of nuclear inclusions. Microvilli and dense cored granules are the most typical and constant structures of Merkel cells (Figure 1-2).

Figure 1-2: Electron micrograph showing a Merkel cell and associated nerve terminal (N) in the hard rabbit-palate. The Merkel cell contains numerous dense cored granules in the cytoplasm at the attaching site of the nerve terminal and has microvilli (arrowheads) extending from the cell surface and desmosomal attachments to neighbouring keratinocytes. Double arrows point to a synapse-like junction between the Merkel cell and nerve terminal (x 10,300). Inset: nuclear inclusion occasionally appears in the Merkel cell nucleus (x 25,000).

(Reproduced from Toyoshima K., et al. In:Suzuki H., Ono T., (eds.) Merkel cells, Merkel cell carcinoma and Neurobiology of the skin. Elsevier Science B.V., Amsterdam, 2000:p6.)

Immunohistochemical studies show that Merkel cells contain properties of both epithelial and neuroendocrine-derived cells. They express namely antigens to the low-molecular weight epithelial cytokeratins (CK) 8, 18, 19 and 20, of which the latter is a well-established marker of mammalian Merkel cells and Merkel cell carcinoma (Moll *et al.*, 1995). Expressed neuroendocrine markers include neuron-specific enolase, chromogranin, synaptophysin and neuropeptide vasoactive intestinal polypeptide but not neurofilament (Haag *et al.*, 1995). Most of these substances are located in the dense cored granules of

Merkel cells. For this reason, it was also suggested that the dense cored granules were the equivalent of synaptic vesicles with the above listed substances serving as neurotransmitters between Merkel cell and nerve terminal. However, none of the substances seem to be implicated in the postulated synaptic transduction process. This leaves the question about the functional role of these substances wide open. So far, there has been no convincing report on exocytosis of these substances from Merkel cells to support the assumption of a paracrine or neuroendocrine role.

Studies of developing human foetuses showed that Merkel cells play a role in the formation of the subepidermal nerve plexus (Narisawa and Hashimoto, 1991; Narisawa *et al.*, 1992; Narisawa *et al.*, 1993), the development of hair follicles, sweat glands and the nerves within the nail bed (Moll and Moll, 1993; Kim and Holbrook, 1995). In adults however, Merkel cells are involved in the deep touch response acting as cutaneous mechanoreceptors. They are closely associated with terminal axons, which directly contact the basal surface of the Merkel cells via fusion with the basal lamina of the epidermis. The cells are found in both hairy and glabrous skin as well as mucous membranes and are concentrated in the skin of acral areas in humans. Although Merkel cells may rarely occur alone, without terminal nerve association in the epidermis or dermis, they are usually found in clusters forming specialized, slowly adapting type I mechanoreceptors such as the tactile hair disc of Pinkus (Haarscheibe) in hairy skin (Pinkus, 1902; Suzuki *et al.*, 2000). The hair discs are considerably elevated above the ordinary skin surface, and each contains one thick hair at its center (Figure 1-3). Under the thick epidermis of the hair discs are numerous Merkel cells. Other specialized structures associated with Merkel cells in humans are the hederiform ending of Merkel-Ranvier found in glabrous skin, nose, lip, palate, and genitalia, and the Merkel touch spots (Tastscheiben) found on the palpebral margin of the eyelid (Winkelmann and Breathnach, 1973; Munger and Halata, 1984).

In all mammalian Merkel cell receptors, the Merkel cells and nerve terminals are always oriented in a way that the Merkel cell faces the direction from which physiological stimuli would normally occur and the nerve terminal on the opposite site (Figure 1-4). Apart from the contact area with the nerve terminal, the surface of Merkel cells forms numerous cytoplasmic processes between surrounding cells. This arrangement suggests that the Merkel cell functions as mechanoelectric transducer with synaptic link to the nerve terminal. This hypothesis is supported by the finding of increases in cytosolic calcium

concentrations within Merkel cells during mechanical stimulation and appears to be essential for the characteristic slowly adapting response of Merkel cell receptors (Baumann *et al.*, 2000; Tazaki *et al.*, 2000). Intensive investigation to identify the neurotransmitter substance, which may be released at this putative synapse, remained negative. As mentioned previously, various candidates had been found immunohistochemically in the dense cored granules of Merkel cells without clear evidence for a neurotransmitter function of any of these substances (Gottschaldt and Vahle-Hinz, 1982).

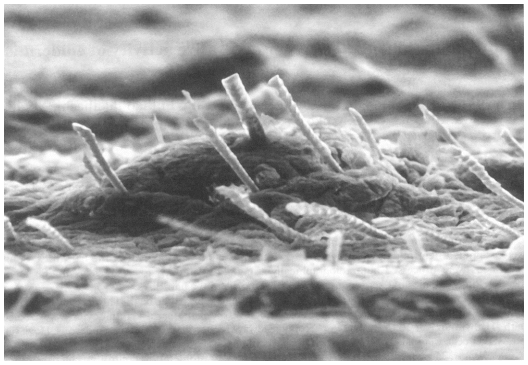


Figure 1-3: Scanning electron microscopy of hair disc in rat abdominal skin. Hair disc is domed structure elevated above the surrounding skin and bears one thick hair near its center.

(Reproduced from Suzuki H., et al. In:Suzuki H., Ono T., (eds.) Merkel cells, Merkel cell carcinoma and Neurobiology of the skin. Elsevier Science B.V., Amsterdam, 2000:p98.)

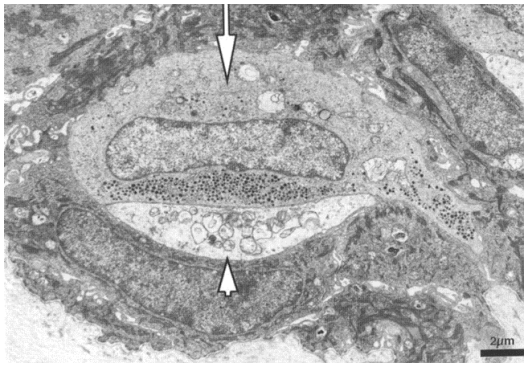


Figure 1-4: Electronmicrograph of a Merkel nerve ending from an epithelial peg of the incisive papilla of a domestic goat. The longitudinal axis of the Merkel cell (arrow) is arranged parallel to the surface of the mucosa (horizontally far outside the picture). The nerve terminal (arrowhead) is positioned below the Merkel cell. The direction of pressure is indicated by the arrow.

(Reproduced from Halata Z. and Baumann K.I., In:Suzuki H., Ono T., (eds.) Merkel cells, Merkel cell carcinoma and Neurobiology of the skin. Elsevier Science B.V., Amsterdam, 2000:p41.)

1.2.1 Merkel cells: developmental origin

Until now, the developmental origin of Merkel cells is still a matter of debate. The presence of both epithelial and neuroendocrine ultrastructural and immunocytochemical features in normal Merkel cells has led to considerable controversy about their origin. According to one view, Merkel cells arise from the neural crest. After separating from Schwann cells they migrate from the mesenchyme through the dermis and into the epidermis, thereby arriving as immigrant cells into the epidermis with extensions of dermal sensory nerves (Hashimoto, 1972). Due to the presence of bioactive amines and peptides in the dense cored granules of Merkel cells, they have been classified as members of the amine precursor uptake and decarboxylation (APUD) system (Winkelmann, 1977). Originally, it was suggested that all APUD cells were derived from the neural crest and therefore Merkel cells were also held as derivatives of the neural crest. Many neuroendocrine cells however can develop from other sources than the neural crest and may be endodermal (Andrew, 1982) or mesodermal in origin (Forssmann *et al.*, 1983). This indicated that a similarity in production of peptide hormones in neuroendocrine cells does not necessarily mean a common developmental origin. These findings together with the observed expression of low-molecular weight simple cytokeratins in Merkel cells contributed to a change in the opinion about their developmental origin. The hypothesis that they are from epidermal origin derived from precursors of keratinocytes (Moll *et al.*, 1986) prevails, at least by some authors, over the neural crest immigration theory. Studies of embryonic and fetal human skin using cytokeratin 20 antibodies demonstrated the occurrence of Merkel cells as early as fetal week 8 (Moll and Moll, 1992). At that time the epidermis starts also to develop epidermal intermediate cells (suprabasal keratinocytes) and Merkel cells were found basally and suprabasally. So the late embryonic/early fetal basal keratinocytes which express both stratified and simple epithelial CKs may be the precursors of suprabasal keratinocytes exclusively expressing stratified epithelial CKs and of Merkel cells expressing only simple epithelial CKs. However, a definitive statement about the origin of mammalian Merkel cells remains difficult. In addition, low-molecular weight cytokeratins were also detected in nonepithelial cells, such as glial cells (Viehweg and Naumann, 1996). The expression of cytokeratins is therefore not necessarily an indication of the epidermal origin of mammalian Merkel cells.

1.2.3 Merkel cell carcinoma: epidemiology and clinical features

Originally, the tumor was believed to be derived from poorly differentiated eccrine sweat glands (Toker, 1972). Subsequent evaluations by immunohistochemistry and electron microscopy showed neurosecretory granules and suggested the Merkel cell to be the cell of origin. A variety of alternative names were then proposed such as primary small cell carcinoma of the skin, murky cell carcinoma and neuroendocrine carcinoma of the skin but the name Merkel cell carcinoma was prevailing based on ultrastructural features similar to Merkel cells (O'Connor and Brodland, 1996).

Since Toker's initial description of this rare skin tumor, less than 2000 cases of MCC have been reported in the world literature to date (Gollard *et al.*, 2000). However, although the true incidence of MCC is unknown, there appears to be an increased prevalence of MCC in regions with a high incidence rate of skin cancer such as Queensland, Australia. MCC predominantly occurs in persons older then 65 years of age, but patients ranging in age from 7 to 95 years have been reported (Hitchcock *et al.*, 1988; Messina *et al.*, 1997). In addition, the tumor has also been described in young adults with congenital ectodermal dysplasia syndromes (Wick *et al.*, 1983; Gherardi *et al.*, 1990). Caucasians are most commonly affected with equal distribution between men and women, but there have been occasional reports in the black population and in Polynesians (Haag *et al.*, 1995). The most frequently involved sites for primary lesions include the skin of the head and neck region (50% or more) of which 20% of these affect the periorbital region and eyelid, followed by extremities (40%) and trunk or mucous membranes (10% or less) (Ratner *et al.*, 1993). The tumor typically presents as a rapidly growing, firm, nontender nodule with a slightly red to violaceous color measuring up to several centimeters in greatest dimension. The overlaying skin is usually intact, smooth, and shiny, although ulceration may be present (Figure 1-5). Occasionally, telangiectases may be found overlying the tumor, mimicking basal cell carcinoma. In its early stages, MCC has a nonspecific appearance resulting in a broad differential diagnosis including undifferentiated squamous cell carcinoma, solid basal cell carcinoma, malignant melanoma, adnexal tumors, adult neuroblastoma, metastatic small cell lung carcinoma and cutaneous malignant lymphoma.

A **B**

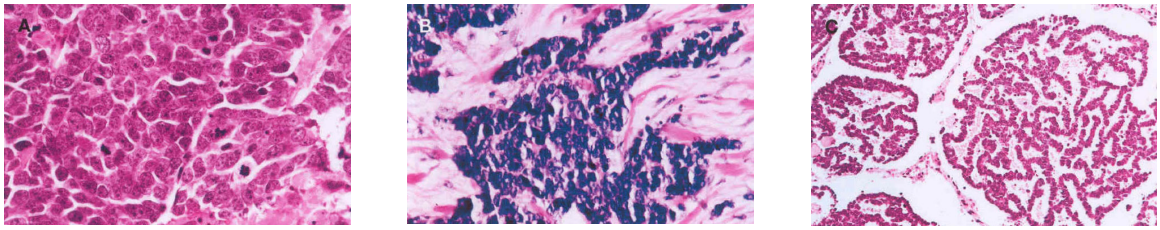
Figure 1-5: Clinical photos of patients with Merkel cell carcinoma on the scalp (A) and the forearm (B). *(Source: Dept. of Dermatology, Ghent University Hospital, Ghent, Belgium)*

The predilection for sun-exposed skin in elderly whites and the fact that MCC can be associated with other UV-induced skin cancers including basal cell carcinoma, squamous cell carcinoma, Bowen's disease and actinic keratoses has implicated prolonged sun-exposure as a possible etiological factor (Pitale *et al.*, 1992). The presence of primary lesions on non sun-exposed sites such as the oral mucosa and genitalia, however, indicates that other etiological factors are also important (Tennvall *et al.*, 1989).

1.2.4 Merkel cell carcinoma: histological features and diagnosis

The vast majority of MCC tumors arises within the dermis and can extend into the subcutaneous fat and muscle tissue. The tumor may rarely involve the overlying epidermis or may appear to be separated from the overlying epidermis by a grenz zone. The tumor cells are round with scanty cytoplasm. In the majority of tumors the nuclei show finely granular dispersed chromatin. Nucleoli are few or absent, very small and usually located adjacent to the nuclear membrane. Mitotic figures are numerous, necrotic and apototic cells are often seen within and around the tumor. Vascular and lymphatic invasion of tumor cells is frequently seen, as well as a surrounding dense infiltrate of lymphocytes and plasma cells (Ratner *et al.*, 1993; Haag *et al.*, 1995).

Based on light microscopy studies the tumor was subdivided into three main histologic subtypes (Gould *et al.*, 1985; Goessling *et al.*, 2002). Most Merkel cell carcinomas represent the "intermediate" cell variant. This variant consists of nodules and diffuse sheets of basophilic cells with minimal cytoplasm and round or oval nuclei (Figure 1-6A). The "small cell" variant is histologically identical to other small cell carcinomas and consists of irregular, hyperchromatic cells, often showing areas of "crushing" artifacts resulting in nuclear molding (Figure 1-6B). The "trabecular" or "classic" variant is the most well-differentiated but is seen in less then one fourth of the cases. This variant is composed of delicate ribbons of small basophilic cells (Figure 1-6C).



A **B** **C**

Figure 1-6: (A) Intermediate variant of MCC showing vesicular, basophilic nuclei with prominent nucleoli and multiple mitoses. (B) Small cell variant, histologically indistinguishable from bronchial small cell carcinoma. (C) Trabecular variant is rarely observed and normally only seen as a small component of a mixed variant. *(Reproduced from Goessling et al., Journal of Clinical Oncology 20: 588-598, 2002)*

The differential diagnosis includes metastatic small cell carcinoma of the lung, a tumor which is often indistinguishable using light microscopy alone. Therefore, electron microscopy and immunohistochemistry play a key role in the correct diagnosis of MCC. The tumor cells of MCC are ultrastructurally similar to normal Merkel cells containing cytoplasmic dense cored granules and bundles of intermediate filaments or paranuclear fibrous bodies. These properties can be sufficient to distinguish MCC from other tumors such as squamous cell carcinoma but most of the time additional immunohistochemical studies are needed for definitive confirmation of MCC (Table 1-1). The markers cytokeratin 20 (CK20) and thyroid transcription factor 1 (TTF-1) are especially useful in distinguishing Merkel cell carcinoma from metastatic small cell lung carcinoma. Positive staining of CK20 is observed in MCC and not in SCLC, while TTF-1 is expressed in SCLC but not in MCC (Leonard *et al.*, 1996a; Cheuk *et al.*, 2001).

Table 1-1: Immunocytochemical differentiation of MCC from other tumors
(Adapted with minor modifications from O'Connor and Brodland, Dermatol Surg., 22: 262-267, 1996)

	Immunocytochemical reactant									
	NSE	*CK8,18,19*	*CK20*	*NFP*	*LCA*	*S100**	*CH A*	*VIP*	*TTF-1*	*CK7*
MCC	+	+ (Paranuclear)	+	+	-	-	+ (50%)	+ (33%)	-	-
SCLC	+	+ (Diffuse)	-	Rare	-	-	+	-	+	+
SCC	-	-	-	-	-	-	-	-	-	-
Melanoma	+	-	-	-	-	+	-	-	-	-
Lymphoma	-	-	-	-	+	-	-	-	-	+ (weak)

NSE, neuron-specific enolase; CK, cytokeratin antigen; NFP, neurofilament protein; LCA, leukocyte common antigen; CH A, chromogranin A; VIP, vasoactive intestinal peptide; TTF-1, thyroid transcription factor-1; MCC, Merkel cell carcinoma; SCLC, small cell lung carcinoma; SCC, squamous cell carcinoma
**A polyclonal antibody is used directed against the αβ dimer form of the S100 protein (S100-A)*

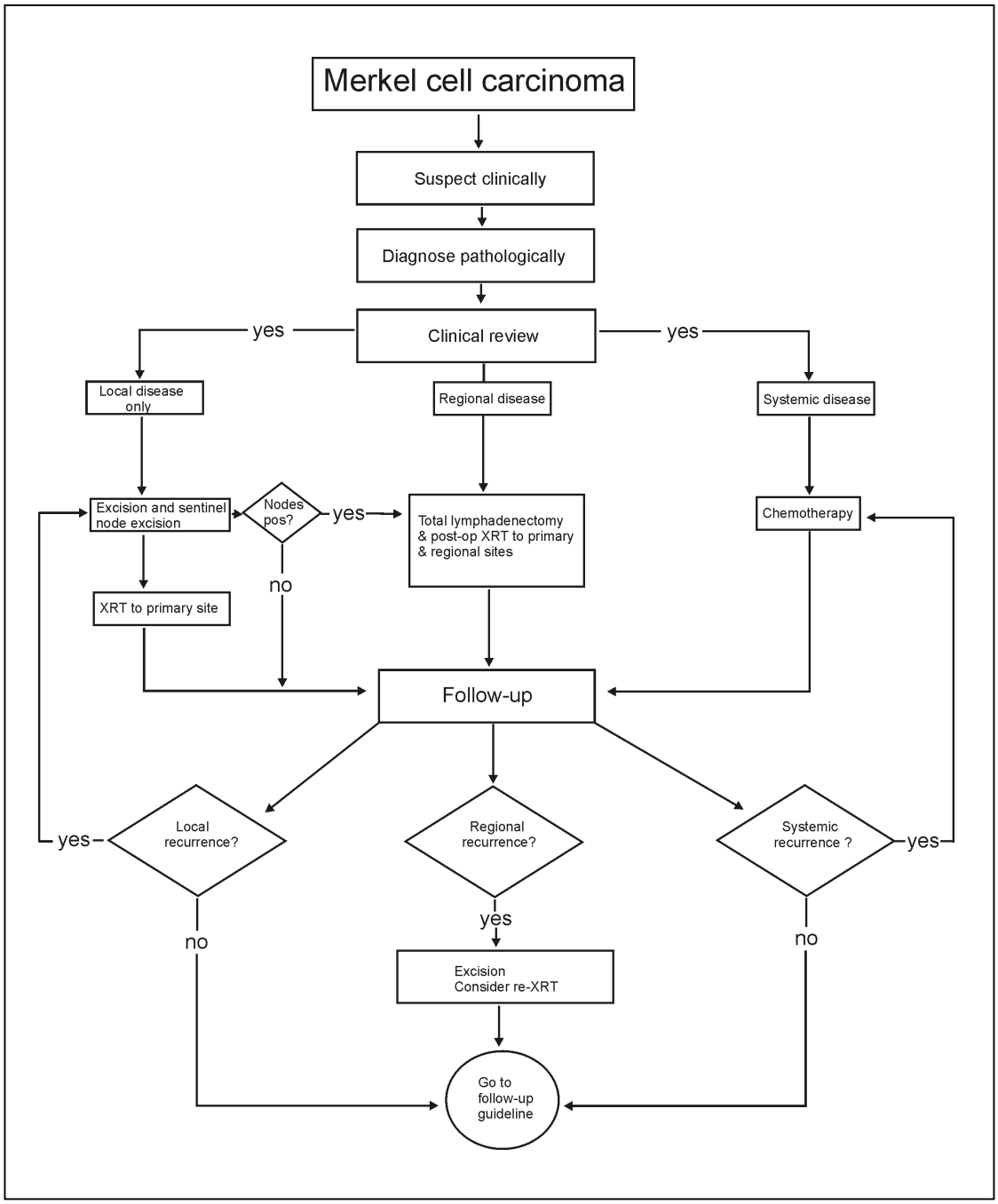
1.2.5 Merkel cell carcinoma: prognosis and treatment

Clinically, MCC is a high-grade, aggressive cutaneous malignancy with a high propensity for local recurrence and regional lymph node metastasis. Specifically, local recurrence develops in 26% to 44% of patients after excision of the primary tumor and is usually apparent within 4 months. Regional lymph node involvement occurs in 55% to 66% of patients and is found at initial presentation in 12% to 31%. The median time between treatment of the primary tumor and clinically detectable nodal metastasis is 7 to 8 months. In patients with nodal involvement, 11% to 66% die of their disease within 5 years. Systemic disease is associated with a particularly poor response. Although rare at initial presentation, one third of patients will develop distant metastases, with the most commonly involved sites being liver, bone, brain, lung and skin. Nearly 50% of the patients followed for 24 months will develop systemic metastases with a mortality rate of 67% to 74% (Smith *et al.*, 2000).

Because of the high degree of local recurrence and early lymph node and distant metastases in patients with MCC, aggressive treatment is given at the time of initial diagnosis. Although no widely adopted classification system exists, treatment guidelines have been based on three clinical stages of disease: local disease without lymph node or systemic involvement (stage I), regional lymph node development without systemic disease (stage II), and systemic metastases (stage III). Most treatment guidelines include wide excision of the primary tumor, alone, or in combination with adjuvant radiation therapy, therapeutic regional lymph node dissection or selective regional lymph node dissection (Ratner *et al.*, 1993). A recently performed study by Smith *et al.* (2000) showed that combining selective lymphadenectomy and irradiation led to improved survival rates compared with radiotherapy alone. In addition, selective lymphadenectomy allowed more accurate treatment with less morbidity to the patient. Chemotherapy is most widely used in patients with systemic disease. Due to the rarity of the condition, no firmly established chemotherapy exists for MCC. The treatment regimens are generally based on those for small cell lung carcinoma. MCC appears initially to respond to various chemotherapy agents including cisplatin and etoposide, but the response is typically short-lived (Redmond *et al.*, 1991; Sharma *et al.*, 1991; Goessling *et al.*, 2002). Table 1-2 presents a treatment guideline for MCC developed at the H. Lee Moffitt Cancer Center in Florida, USA.

Table 1-2: Treatment guideline for Merkel cell carcinoma

(Reproduced from Smith et al., Cancer Control 7:72-83, 2000)



Surprisingly, a few cases of MCC showing complete spontaneous regression have been reported in the literature (Connelly *et al.*, 2000; Maruo *et al.*, 2000). Since its first description by O'Rourke and Bell, (1986) the mechanism of spontaneous regression in MCC is unknown and partially hampered by the lack of long-term follow-up studies of previously published cases. A recent report by Maruo *et al.* (2000) described the complete clinical course of an 80-year-old Japanese woman with regressing MCC, including histopathological analysis of the tumor just before complete disappearance. The results revealed that the tumor cells were completely replaced by numerous foamy cells, similar to a previously described case with spontaneously regressing malignant melanoma (Kanzaki and Nishiyama, 1985). The authors speculated that in malignant melanoma, tumor cells might be killed by humoral immunity-mediated membrane lysis in the early stage and by cell-mediated vacuolization in the late stage of spontaneous regression, while macrophages finally replace the tumor cells. The same phenomenon could occur in regressing MCCs although further detailed investigations will be necessary to support this hypothesis. Nevertheless, Merkel cell carcinoma remains an extremely aggressive tumor not underestimating the importance of an early diagnosis and aggressive treatment.

1.3 Genetics of Merkel cell carcinoma

1.3.1 Cytogenetic studies

In contrast to other more common tumor types, the available cytogenetic data for MCC are sparse. A total of 37 cases have been examined of which eight cases were analyzed in this study (Table 1-3). One of the major advantages of karyotyping is that the entire genome is analyzed within one analysis. By this method not only gains and losses of chromosomal segments can be detected but also the chromosomal rearrangements (e. g. unbalanced translocations) which lead to these unbalances. Karyotyping is also essential in the detection of balanced chromosomal rearrangements which may lead to oncogene-activation or fusion genes. One of the most striking findings of cytogenetic studies of MCC was the occurrence of structural rearrangements involving the short arm of chromosome 1 in almost 40% of the investigated cases, often leading to loss of chromosomal region 1p36. This chromosomal region is often deleted in a wide variety of neoplasms including neuroendocrine tumors such as neuroblastoma and melanoma but also other tumors such as breast carcinoma, colon carcinoma and hepatocellular carcinoma. Several candidate tumor suppressor genes have been mapped to 1p36 but thusfar none of the investigated candidates seemed to be directly involved in tumors with 1p loss (Schwab *et al.*, 1996; Judson *et al.*, 2000; Steele-Perkins *et al.*, 2001).

Table 1-3: Overview of the reported chromosomal abnormalities in MCC

Reference	Case	Tumor type[a]	Modal number	Structural and numerical abnormalities
Kusyk *et al*., 1986	1	LNM	54-64	+X,del(1)(q?23q?24)x2,i(13q),der(15)t(15;21)(q13;q25), +6, +7,+9,+10,+11,+18,+19,+20,+mar
Hopfner *et al*., 1987	2	P	47	i(8q),+6,-10,+12
Rosen *et al*., 1987	3	CL	47-48	der(1)t(1;17)(p36;q21),i(5p)x2,der(7)t(7;8)(q26;q22)
Sozzi *et al*., 1988	4	P	45	inv(1)(p33q11),+del(1)(p22p33),+1
	5	LNM	48	der(1)t(1;?)(p36;?),+1,+3,+6
	6	LNM	49	i(7p),del(11)(p13p14),der(15)t(15;?)(p12;?),+6,+11,+12
	7	LNM	44	del(1)(p22p32)(q41q44),del(3)(q13q29),+1
	8	P, LNM	54	del(1)(q31q44),t(1;3)(q31;q26),+1,+9,+10,+11,+12,+14,+18, +19,+20,+21,+4mar
Sandbrick *et al*., 1988	9	LNM	48	del(7)(q31.2),+6,+11
Shabtai *et al*., 1989	10	LNM	46	del(2)(p11),del(22q)(q12),-5,-14,-17,+20,-22
Koduru *et al*., 1989	11	LNM	46	der(1)t(1;13)(q21;p13),del(5)(q31)
	12	LNM	46	i(1q),der(2)t(2;?)(q37;?),i(6p),der(10)t(10;?)(p15;?),der(17)t (17;?)(p13;?),-4,+mar
	13	P, LNM	47	der(1)t(1;6)(q22;p25),der(21)t(21;?)(p13;?),+12,-20
English *et al*., 1990	14	LNM	47	+7 or add(7)(q?)
Smadja *et al*., 1991	15	BMM	46	dup(1)(q11q44),t(2;3)(p13;q?26q?27),der(15)t(15;?)(p12;?)
Ronan *et al*., 1993	16	CL	46	t(1;17)(p36;q21),t(1;6)(p22;q26),r(8),+2,-8,-17
Leonard *et al*., 1993	17	LNM	43	der(1)t(1;5)(p36;p13),i(11p)(p10),-1,-11,-13,-22,+mar
	18	REC	49	+X,-Y,add(7)(p22),i(9q),i(11p),-7,-9,-11,+14
	19	P	44	del(1)(p22),del(1)(q21),der(1)(1;?)(q21;?),-1,-3,-11,-13, -13,+18
	20	LNM	55	+1,-8,+7mar
	21	REC	47-49	+1,-5,-8,-13,-13,+5mar
	22	REC	43-44	-9,-13,-22
Schlegelberger *et al*., 1994	23	LNM	46-48	der(X)t(X;10)(p22;q23),t(1;15)(q12;p13),t(1;16)(q21;p13), t(1;14)(p12;q32),add(10)(p11),+18,+19
Gibas *et al*., 1994	24	P	46	del(1)(p36.1)
Tope *et al*., 1994	25	P	46-48	-Y,+5,+7,+18

Table 1-3 (continued)

Moll *et al*., 1994	26	CL	45-50	i(5p)x2,add(1)(p?),t(1;12)(p13;q13),del(1)(p?),del(2)(q33), add(6q),add(12q)
	27	CL	49	del(11)(q21),t(5;17)(p15;p13),t(1;7)(q32;p15p),+6,+20,+Y
Perlman *et al*., 1995	28	PM	46	der(1)t(1;3;22)(p34;q28→q11;q12),der(3)t(1;3)(p35;q11), der(22)t(3;22)(q29;q11)
Vazquez-Mazariego *et al*., 1996	29	LNM	46	der(6)t(1;6)(q23;q27),der(9)t(1;9)(q11;p24)
Larsimont *et al*., 1996	30	P	47	+6
Van Gele *et al*., 1998	31	P	46	der(1)t(1;1)(p36.31;q12)
	16	CL	47	ins(1;?)(p36.2;?),ins(6;?)(q15;?),dic(8;8)(8qter→8p21.2::8p 21.2-->8qter),+r?
Van Gele *et al*., accepted	32	CL	46	t(7;8;14)(7pter→7q11.23::14q31.1→qter;8pter→8q23::7q11. 23→7q31.1::8q23→8qter;14pter→14q31.1::7q31.1→7qter), der(9)(9pter→9q34::13q22.2→qter),der(13)(13pter→13q12. 3::16q23q24→16qter),del(12)(q12q14)
	33	CL	60	+der(X)(Xpter→Xq?::16p?q?→16p?q?),+der(1)(1pter→1q23 ::3q23→3qter),del(2)(pter→p13::p16→qter),+der(2)(:2p24;9 qter→9q12),der(3)(3pter→3q23::7q21→7qter),der(4)(1qter →1q12::4p16→4qter),der(5)(5p15;11qter→11q14.2),+der (5)(5pter→5q12;18qter→18q?),+der(5)(5pter→5q12;22p?q?),+6,+8,del(11)(q14.2),+del(12)(q12),+12,der(14)(8p?q?→8p ?8q?::Xq?→Xq?::14p11.1→14qter),+15,+18,der(19)t(19;20) (p13.3;p?q?),+20,+20,+der(21)(21qter→21p11.2::14q12→ 14qter),+22
	34[b]	CL	48	i(5)(p10)x2,der(7)t(7;11)(q36,q14)
	35[b]	CL	46	der(1)t(1;17)(p36.3;q21.3),8,der(10)t(10;12)(q23;p11.21)
	36	CL	41	der(1)(3qter→3q13.3~21::1p35.1→qter),der(3)(3pter→3q 13.3~21::1p36.1→1pter),3,i(4)(p10),der(6)(6pter→6q16;8q2 2→8qter),-10,der(11)(11pter→11q24::3q23→3q25-26.1:: 13q?→13q?::3q?29→3qter),-13,der(14)(21qter→21q21:: 3p?3q?→3p?q?::13q?→13q?::14p11.2→14qter),der(16)(16 pter→16q24::13q?→→13qter),der(17)(Yqter→Yp11.2::17q1 1.1→17qter::1p35.1→1p34.3::3q13.3~21→3qter),der(20)(20 pter→20q13::4q13→4qter),-21,der(22)(22pter→22p11.2:: 3q11.2::22p11.2→22qter)
	16	CL	45-46	ins(1;?)(1pter→1p36.2::1q12::?::1p36.2→1qter),ins(6;1;?)(6 pter→6q15::1q12::?::6q15→6qter),dic(8;8)(8qter→8p21.2::8 p21.2→8qter),der(1)r(1;?)(q12::?)
	37	M, LNM	45	t(3;9;15;17;22)(3pter→3q13.1::22q12→22qter;9pter→9q13:: 3q13.1→3q25-26.1::9q13→9q34::15q21.1→15qter;15pter →15q21.1::3q25-26.1→3qter;17pter→17q24::9q34→9qter; 22pter→22q12::17q24→17qter),-10

[a]LNM; Lymph Node Metastasis, P; Primary tumor, CL; Cell Line, REC, Recurrence at site, PM; Pulmonary Metastasis, M; Metastatic tumor

[b]Cases 34 and 35 are subclones of the originally described cell line MKL-1 by Rosen *et al*., 1987

1.3.2 Molecular cytogenetic studies

Molecular cytogenetic analyses have been widely applied in the study of cancer genetics. However, at the start of this study this methodology has not been exploited to investigate MCC. Therefore, two whole genome scanning methods with proven complementary resolving power (Van Roy *et al.*, 2001; Van Limbergen *et al.*, 2002) were used in this study. Firstly, we applied a technique called 'comparative genomic hybridization' (CGH) which enables the whole genome to be screened for chromosomal losses, gains and gene amplifications in one single experiment. We analyzed 26 tumor samples and eight cell lines by CGH. This study revealed a characteristic pattern of chromosomal gains and losses occurring in MCC (see Part 2). A second approach was the use of multiplex FISH (Speicher *et al.*, 1996) in combination with CGH and karyotyping. This technique allows hybridization of 24 differentially labeled chromosome-specific painting probes onto tumor metaphase spreads. In contrary to conventional FISH, prior knowledge of the nature of chromosomal rearrangements is not required. In addition, M-FISH allows detection of balanced translocations which is not possible with CGH. By combined analysis of six MCC cell lines and one MCC tumor a detailed characterization of previously unnoticed (complex) rearrangements was obtained. The results of this study are described in Part 2.

1.3.3 Loss of heterozygosity (LOH) studies and candidate gene analysis

In LOH studies highly polymorphic markers such as microsatellites (Weissenbach *et al.*, 1992) or single nucleotide polymorphisms (SNPs) (Sherry *et al.*, 2001) are used to detect small deleted regions by comparing the genotype of a tumor to that of its matching normal DNA. Shortest regions of overlap (SROs) of deleted regions are defined which can point to the possible presence of one or more putative tumor suppressor genes in this particular region (Devilee *et al.*, 2001). So far there have been only a few LOH studies performed in the search for possible candidate tumor suppressor genes involved in MCC. Three LOH studies on the short arm of chromosome 1 showed frequent occurrence of 1p deletions and resulted in the delineation of multiple SROs, indicating that more than one tumor suppressor gene is likely to play a role in the development of MCC (Harnett *et al.*, 1991; Vortmeyer *et al.*, 1998; Leonard *et al.*, 2000) (Table 1-4). A targeted analysis of two 1p36 breakpoints was performed by FISH with region specific probes and polymorphic markers

in this study. This led to the identification of two distinct regions, 1p36.2 and 1p36.3 respectively being involved in MCC (see Part 2). A possible candidate tumor suppressor gene for MCC tumors with distal 1p36 deletions was the *TP73* gene. Mutation analysis of this gene together with *TP53* was performed for MCC in this study resulting in one *TP73* missense mutation and several *TP53* mutations (see Part 3 and Table 1-4). Other LOH studies to search for deletions at 3p, 13q, 10q and 9p have been performed together with mutation analysis or expression studies of candidate tumor suppressor genes such as *FHIT*, *RB1*, *PTEN* (see Part 3), *p16*^{INK4a} and *p14*^{ARF} located in these deleted regions (Table 1-4).

Table 1-4: Overview of reported LOH and molecular analysis studies in MCC

References	Number of cases	Chromosomal region	SRO at	Candidate gene analysis: Gene: Results:
Harnett *et al*., 1991	3 tumors	1p	1p35-36	Not done
Leonard *et al*., 1996b	26 tumors	3p	3p13-21.1	Not done
Sozzi *et al*., 1996	14 tumors	3p14.2	Not applicable	*FHIT* gene: - abnormal FHIT products in 57% MCCs lacking three or more exons
Leonard and Hayward, 1997	24 tumors	13q	13q14.3	*RB1* gene: - no detectable RB1 expression by Western blot analysis
Schmid *et al*., 1997	25 tumors	17p	Not applicable	*TP53* gene: - TP53-immunoreactive nuclei in 20% MCCs but no mutations in exons 4-8 by SSCP
Vortmeyer *et al*., 1998	10 tumors	1p	1p35-36	Not done
Van Gele *et al*., 2000	10 tumors and 5 cell lines	1p 17p	Not applicable Not applicable	*TP73* gene: - one missense mutation *TP53* gene: - four missense and one non-sense mutation by direct sequencing of cDNA
Leonard *et al*., 2000	22 tumors and 6 cell lines	1p	1p36 1p35 1p32-33	Not done
Van Gele *et al*., 2001	26 tumors and 8 cell lines	10q23	10q23.3	*PTEN* gene: - one nonsense mutation - one homozygous deletion of exon 9
Cook *et al*., 2001	29 tumors and 5 cell lines	9p	9p24 9p21	*p16*^{INK4a} and *p14*^{ARF} genes: - no mutations - expression of both proteins detected by Western blots and immunohistochemical studies
Popp *et al*., 2002	6 cell lines	17p	Not applicable	*TP53* gene: - two missense mutations by sequencing of exons 5-8

1.3.4 Genetic similarities and differences between MCC, SCLC, melanoma, SCC and BCC

Unlike SCLC, only few CGH studies have been performed for MCC, cutaneous malignant melanoma, squamous cell carcinoma (SCC) of the skin and basal cell carcinoma (BCC). Table 1-5 gives an overview of the most frequently chromosomal gains and losses detected by CGH in at least one of the above mentioned tumor types. These data illustrate that the most common genetic changes are found between MCC and SCLC.

Table 1-5: Presence (+) or absence (-) of gains and losses detected by CGH in MCC, SCLC, SCC, melanoma and BCC[a]

	MCC[c]	SCLC[d]	SCC[e]	Melanoma[f]	BCC[g]
1q+[b]	+ WCG	-	-	+	-
+2	-	-	-	+	-
3p-	+	+	+	-	-
3q+	+	+	-	-	-
4p-	+	+	+	-	-
4q-	-	+	-	-	-
5p+	+	+	-	-	-
5q-	+	+	-	-	-
6p+	+ WCG	-	-	+	+
6q-	-	-	-	+	-
7p+	+ WCG	-	+	+ WCG	+ WCG
8p-	+	+	+	+	-
8q+	+	+	+	+ WCG	-
9p-	-	-	+	+	-
9p+	-	-	-	-	+
9q-	-	-	-	-	+
10q-	+ WCL	+	-	+ WCL	-
11q-	+	-	-	-	-
11q+	-	-	+	-	-
13q-	+	+	-	-	-
17p-	+	+	-	-	-
17q+	-	+	-	+ WCG	-
+19	+	-	-	-	-
+20	+	-	-	+	-
Xq+	+ WCG	+	-	-	+ WCG

[a]Grey boxes are used to facilitate visual inspection of similarities between MCC and other tumor types; WCG, whole chromosome gain; WCL, whole chromosome loss

[b]Representation of frequently occurring chromosomal abnormalities in MCC, SCLC, SCC, melanoma and/or BCC based on CGH reports

Results based on CGH studies of [c]Van Gele *et al*., 1998 and Popp *et al*., 2002; [d]Ried *et al*., 1994, Levin *et al*., 1995 and Petersen *et al*., 1997; [e]Popp *et al*., 2002; [f]Bastian *et al*., 1998 and Balázs *et al*., 2001; [g]Ashton *et al*., 2001

In addition to gains and losses, distinct high-level amplifications are often observed in SCLC, SCC and melanoma whereas gene amplifications are rarely seen in MCC and BCC.

1.4 Aims of the study

The aim of this thesis was to make a contribution to the understanding of the genetic basis of Merkel cell carcinoma. To this purpose three strategies were applied:

1. ***Molecular cytogenetic identification of critical genomic regions in Merkel cell carcinoma.***

   In view of the frequent occurrence of 1p abnormalities in MCC, we applied FISH with region specific probes and microsatellite markers to study two MCC cases carrying 1p36 rearrangements in order to delineate the position of putative tumor suppressor gene(s). To search for other critical chromosomal regions implicated in MCC various whole genome screening methods were used. Standard karyotyping was performed on two MCC tumors and six MCC cell lines. Twenty-six tumor samples and eight MCC cell lines were investigated by CGH to search for characteristic patterns of chromosomal losses, gains and gene amplifications. M-FISH in combination with karyotyping, conventional FISH and CGH was applied in order to describe complex chromosomal rearrangements in further detail. The results of these studies are reported in Part 2.

2. ***Mutation analysis of candidate tumor suppressor genes in Merkel cell carcinoma.***

   Mutation analysis was performed for (candidate) tumor suppressor genes *TP73* (1p36.3) and *PTEN* (10q23.3) both located in chromosomal regions which were shown to be recurrently lost by one or more of the above mentioned techniques. The results of these studies are described in Part 3.

3. ***Differential gene expression analysis in Merkel cell and small cell lung carcinoma.***

   In view of the current limited understanding of the molecular pathogenesis of MCC and the clinical, morphological and genetic similarities with SCLC, we performed gene expression profiling analysis of 10 MCC and 4 SCLC cell lines using Atlas cDNA expression arrays containing 1891 unique genes involved in many cellular functions. We searched for biological markers to improve differential diagnosis of MCC and SCLC. In addition, we aimed to identify specific gene expression signatures associated with the phenotypically different subgroups of MCC. The results of this study are described in Part 4.

PART 2

Identification Of Critical Genomic Regions

2.1 Molecular cytogenetic identification of critical genomic regions

2.1.1. Molecular analysis of 1p36 breakpoints in two Merkel cell carcinomas

Van Gele M., Van Roy N., Ronan S.G., Messiaen L., Vandesompele J., Geerts M.L., Naeyaert J.M., Blennow E., Bar-Am I., Das Gupta T.K., van der Drift P., Versteeg R.,. Leonard J.H, and Speleman F. *Genes Chrom Cancer*, *23: 67-71 (1998).*

2.1.2. Characteristic pattern of chromosomal gains and losses in Merkel cell carcinoma detected by comparative genomic hybridization

Van Gele M., Speleman F., Vandesompele J., Van Roy N., and Leonard J.H.
Cancer Res, 58: 1503-1508 (1998).

2.1.3. Combined karyotyping, CGH and M-FISH analysis allows detailed characterization of unidentified chromosomal rearrangements in Merkel cell carcinoma

Van Gele M., Leonard J.H., Van Roy N., Van Limbergen H., Van Belle S., Cocquyt V., Salwen H., De Paepe A., and Speleman F. *Int J cancer (accepted).*

2.1.1. Molecular analysis of 1p36 rearrangements in Merkel cell carcinomas

Our initial interest in MCC was triggered by reports describing 1p abnormalities, often leading to loss of distal 1p material and a paper by Ronan *et al*. (1993) describing an unbalanced 1;17 translocation. As both 1p deletions and 1;17 translocations were frequently recognized in neuroblastoma, a pediatric neuroectodermal tumor, a pilot study was set up in order to study 1p abnormalities in MCC.

In this report, FISH with region specific probes and high-polymorphic microsatellite markers were used to study two particular chromosome 1p36 rearrangements in an MCC tumor and MCC cell line, respectively. Our analysis showed that two distinct regions, 1p36.3 and 1p36.2, can be implicated in MCC. Each of these regions could harbour a putative tumor suppressor locus. Our findings provided thus further evidence for a role of at least two tumor suppressor genes on 1p involved in MCC.

GENES, CHROMOSOMES & CANCER 23:67–71 (1998)

BRIEF COMMUNICATION

Molecular Analysis of 1p36 Breakpoints in Two Merkel Cell Carcinomas

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Merkel cell carcinoma (MCC) is a rare aggressive neuroendocrine tumor of the skin. Only little information is available on the genetic alterations occurring in this tumor. Cytogenetic studies thus far have not shown recurrent chromosomal changes, although various structural chromosome 1 rearrangements, including deletions, often leading to loss of distal 1p material appear to be frequent. We report on fluorescence in situ hybridization and loss of heterozygosity analyses of an MCC tumor and MCC cell line UISO. The present study has shown that two distinct regions in the most distal band 1p36 on the short arm of chromosome 1 can be implicated in MCC. One region at 1p36.3 was delineated by a distal deletion in the MCC tumor as a result of an unbalanced translocation, resulting in loss of all markers distal to *ENO1*. This region was previously shown to be deleted in different tumor types including neuroblastoma. In cell line UISO an insertion in 1p36.2 was identified. The insertion breakpoint indicates a second, more proximal, region on 1p involved in MCC. The insertion breakpoint was mapped within a cluster of repetitive tRNA and snRNA genes and thus could coincide with the constitutional 1p36 breakpoint previously reported in a patient with neuroblastoma. *Genes Chromosomes Cancer 23:67–71, 1998.*

Merkel cell carcinoma (MCC), or neuroendocrine carcinoma, is a rare tumor of the skin that was first described by Toker in 1972. The tumor is thought to be derived from Merkel cells, which are located in the basal epidermis in areas of tactually sensitive skin. They are thought to be mechanoreceptors of neuroendocrine origin, although their exact function is still controversial. The disease occurs predominantly in older fair-skinned people. To date, karyotypic analysis on MCC has been performed on 24 tumors and five cell lines. Only three loss of heterozygosity (LOH) studies have been reported thus far (Harnett et al., 1991; Leonard et al., 1996; Leonard and Hayward, 1997). All of these studies showed a variety of chromosomal abnormalities. No particular recurrent chromosome translocation has been described, but structural rearrangements involving chromosome 1 have been frequent. Often, 1p rearrangements, including deletions, were observed. Loss of chromosome arm 1p material has also been described in many other tumors including melanoma, colon carcinoma, breast carcinoma, hepatocellular carcinoma, pheochromocytoma, and neuroblastoma (for review, see Schwab et al., 1996). These observations strongly suggest the presence of tumor suppressor genes on 1p that are implicated in the development of these tumors. Combined cytogenetic and molecular analyses in neuroblastoma allowed us to propose at least three distinct putative tumor suppressor loci on 1p (Versteeg et al., 1995). In the present study, we used a similar approach for MCC and found two chromosome 1 rearrangements, each of which could point at distinct chromosomal regions harboring putative tumor suppressor loci.

Patient UHG-VM was a woman born in 1915. She was first seen in the Department of Dermatology in June 1995 with a preliminary diagnosis of B-cell lymphoma. Clinically, she had a rapidly growing tumor on the right lower calf, with two palpable lymph node metastases in the right inguinal region. Biopsies from the primary tumor and of the pathologically enlarged inguinal lymph nodes were diagnosed as MCC by histological examination. Immunohistochemistry showed positivity for

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NSE and CAM 5.2 (paranuclear dots). Staging procedures included computed tomographic examinations of the chest, abdomen, and brain and bone marrow examination. No internal tumors or metastases were detected. The general clinical condition of the patient was poor, and treatment was started with photon therapy of the primary tumor and the right inguinal region. During this therapy, multiple in-transit metastases developed and treatment was changed to electron beam therapy (cumulative dose of 60 Gy). The volume of the primary tumor regressed about 40%. The patient died in October 1995.

Karyotyping was performed on G-banded metaphase cells obtained from short-term cultures of tumor UHG-VM according to standard procedures. The karyotype of cell line UISO has been described by Ronan et al. (1993). Fluorescence in situ hybridization (FISH) was performed according to the method of Van Roy et al. (1994) by using the following DNA probes: chromosome-specific plasmid (pBS) libraries for all individual chromosomes, pUC1.77 (D1Z1 for the heterochromatic region of chromosome 1), p1–79 (D1Z2 on 1p36.33), c102 (D1S172 on 1p36.33), p58 (*CDC2L1* on 1p36.33, Oncor P5124-dig), λ27 (*ENO1* on 1p36.32-p36.31), 12A-2 (*NPPA* on 1p36.31-p36.23), and cHE2.6 (A12M2 on 1p36.1). PACs LLNLP704N14224Q and LLNLP704I09167Q were shown to flank the 2-Mb cluster of tRNA and snRNA genes at 1p36.2 (van der Drift, personal communication). The following CEPH YAC clones for the 1p region were used: 807h7 (*RIZ* on 1p36), 209e9 (*MYCL1* on 1p34.3), and 728f1. The YAC clone 728f1 shows three distinct hybridization patterns on chromosome 1 (1p36.2-p36.13, 1p12, and 1q21) due to the presence of multiple tRNA and snRNA genes and pseudogenes at these sites (unpublished data). FISH images were recorded on a Leitz DM microscope with a black-and-white CCD camera (Sony IMAC-CCD 830) and the ISIS software program from Metasystems (Germany) for image capture and digitization. Color prints were made on a digital color Mitsubishi printer CP-D1E. LOH analysis was performed by using the following microsatellite CA-repeat markers for the short arm of chromosome 1: D1S243, D1S214, D1S228, D1S199, D1S234, D1S211, D1S220, and D1S216. The CA-strand primers were fluorescein labeled at the 5′ end. CA-repeat fragments were amplified essentially as described by Schleiermacher et al. (1994). All polymerase chain reaction fragments were electrophoresed on an ALF™; Automated DNA sequencer (Pharmacia) on a 6% LongRanger™; (AT Biochem) 8 M urea denaturing gel. In every lane, size markers (63, 135, 250, and/or 334 bp) were loaded to correct for minor bandshifts during gel electrophoresis.

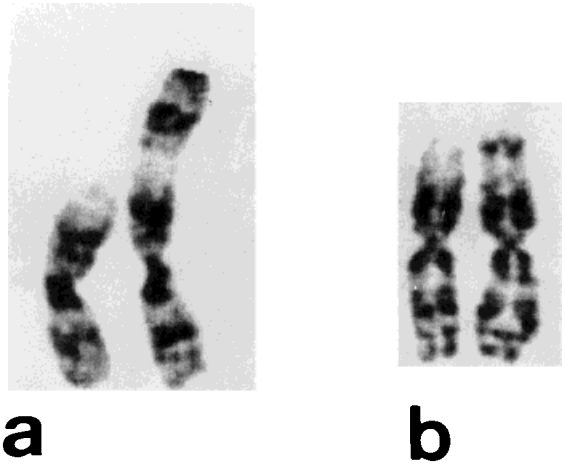

Figure 1. Partial G-banded karyotypes showing the chromosomes 1 of tumor UHG-VM (**a**) and cell line UISO (**b**).

Genotyping for all markers was carried out by using the Fragment Manager™; 1.1 software.

Karyotype analysis performed on short-term cultures of MCC tumor UHG-VM showed a 1p rearrangement as the only structural chromosomal change. Additional material was translocated onto the short arm of one chromosome 1. The banding pattern of this additional material suggested a 1q origin (Fig. 1a), which was confirmed by FISH with a chromosome 1 library (not shown). Cohybridization of pUC1.77 (D1Z1) and p1–79 (D1Z2) demonstrated the presence of D1Z1 sequences in the translocated 1q segment, indicating that an entire chromosome 1 long arm was translocated. This hybridization also showed loss of the D1Z2 locus on the der(1) (Fig. 2a). Subsequent hybridizations with 1p-specific probes showed that the deleted segment was small because only the most distal markers D1S172, CDC2L1, and D1Z2 were lost. Because *ENO1* was retained, the deleted segment did not extend further than subband 1p36.31 (Fig. 3). LOH analysis was in agreement with the data obtained by FISH (Fig. 3). Thus, the karyotype is described as 46,XX,der(1)t(1;1)(p36;q12–21).ish der(1)t(1;1)(p36.31;q12) (wcp1+,D1Z1++, D1S172−, CDC2L1−, D1Z2,ENO1+, NPPA+, A12M2+, MYCL1+).

In the MCC cell line UISO, the abnormal chromosome 1 was originally described as an unbalanced translocation t(1;17)(p36;q21) together with other cytogenetic abnormalities (Ronan et al., 1993). The derivative chromosome 1 is shown in Figure 1b. Cohybridization of libraries specific to chromosomes 1 and 17 could not demonstrate the presence of chromosome 17 material on the der(1) (not

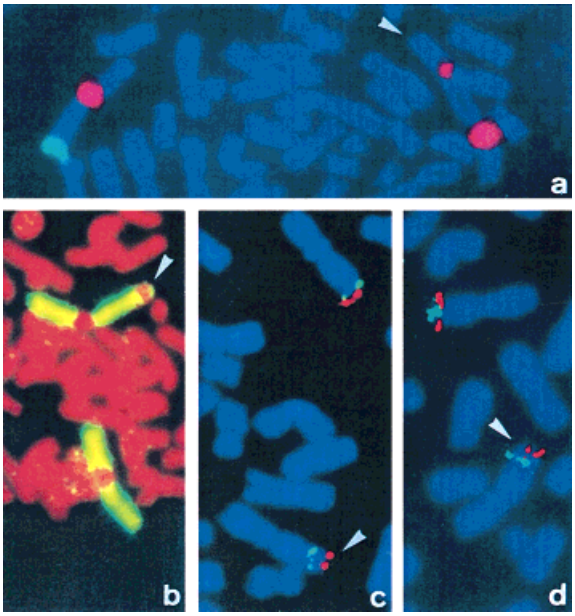

Figure 2. FISH on tumor UHG-VM using probes for region 1q12 (D1Z1, red) and 1p36.33 (D1Z2, green; **a)** and cell line UISO using probes for chromosome 1 (pBS-1; **b**), probes for loci D1Z2 (red) and A12M2 (green; **c**), and probes for PAC clones LLNLP704N14224Q (red) and LLNLP704I09167Q (green) flanking the repetitive tRNA–snRNA gene cluster on 1p36.2. (**d**). Arrowheads point at the abnormal chromosome 1.

shown). The staining pattern of the chromosome 1 library indicated that the der(1) was the result of an insertion rather than an unbalanced translocation (Fig. 2b). To confirm the presence of the insertion and to determine the insertion breakpoint, a panel of 1p probes was tested with D1Z2 by two-color hybridization. Overlap or close colocalization of both probes on the der(1) indicated that the tested DNA probe mapped distal to the insertion breakpoint. Separation of both signals indicated that the tested DNA probe mapped proximal to the insertion breakpoint. Using this approach, we initially mapped the 1p breakpoint between *RIZ* and A12M2 (Fig. 2c). A12M2 has been shown to map within a 2-Mb tRNA–snRNA gene cluster on 1p36.2 (van der Drift et al., 1994). Hybridization with YAC 728f1 showed that this gene cluster was split because of the insertion (not shown). This observation was confirmed by cohybridization with two PAC clones flanking the tRNA–snRNA gene cluster (Fig. 2d). All tested 1p markers were present in one copy on the der(1), and LOH markers (Fig. 3) were found to be retained, indicating that no detectable loss of 1p material resulted from the insertion at 1p36.2. In a first attempt to identify the chromosomal origin of the inserted material, we hybridized metaphases from this cell line with libraries from each individual human chromosome, but no specific staining at 1p36 could be observed. Further attempts with microdissection and SKY analysis also remained inconclusive.

In the MCC tumor, cytogenetic and FISH analyses demonstrated translocation of an entire chromosome 1 long arm onto the distal end of the short arm (1p36) of a chromosome 1 as the sole karyotypic change. Gibas et al. (1994) also reported a 1p36 abnormality as the only chromosomal change in an MCC tumor. Furthermore, loss or structural rearrangements of band 1p36 with other karyotypic abnormalities occurs frequently in MCC. Together with the literature data, the present observation provides further evidence for a role of one or more genes on 1p in the development of MCC. Recently, LOH for one or more 1p loci was observed in 75% of MCC tumors (Leonard et al., unpublished data).

In MCC tumor UHG-VM, FISH using region-specific probes and LOH analysis showed loss of the most distal part of 1p (1p36.31 → pter) including markers D1Z2, CDC2L1, and D1S172. The analysis of cell line UISO showed an insertion at 1p36.2. This insertion breakpoint was located proximal to the deleted region in tumor UHG-VM and between the *RIZ* gene, which encodes for a retinoblastoma binding protein (Buyse et al., 1995) and the adenovirus integration site (A12M2), which is located within a cluster of tRNA–snRNA genes (van der Drift et al., 1994). Molecular analysis of the MCC tumor UHG-VM and MCC cell line UISO thus provides evidence for involvement of two distinct loci within 1p36.

Loss of chromosome arm 1p material has also been reported in many other tumors including neuroblastoma. In neuroblastoma, a tumor of neuroendocrine origin as is MCC, several putative tumor suppressor loci on 1p have been proposed. An imprinted tumor suppressor gene is thought to reside in a frequently deleted region in 1p36.3 delineated by loci D1S47 (telomeric) and D1S244 (centromeric) (Caron et al., 1995; Schwab et al., 1996). The monoallelically expressed *TP73* gene, which is a *TP53* homologue, maps within this region (Kaghad et al., 1997). One of the more proximal putative neuroblastoma tumor suppressor loci is delineated by the 1p breakpoint of the constitutional 1;17 translocation found in a patient with neuroblastoma and coincides with the insertion breakpoint of the MCC cell line UISO (Laureys et al., 1995; van der Drift et al., 1995). Cloning of the constitutional 1;17 breakpoint and the UISO 1p36 insertion breakpoint will resolve whether the same gene in both rearrangements is affected and

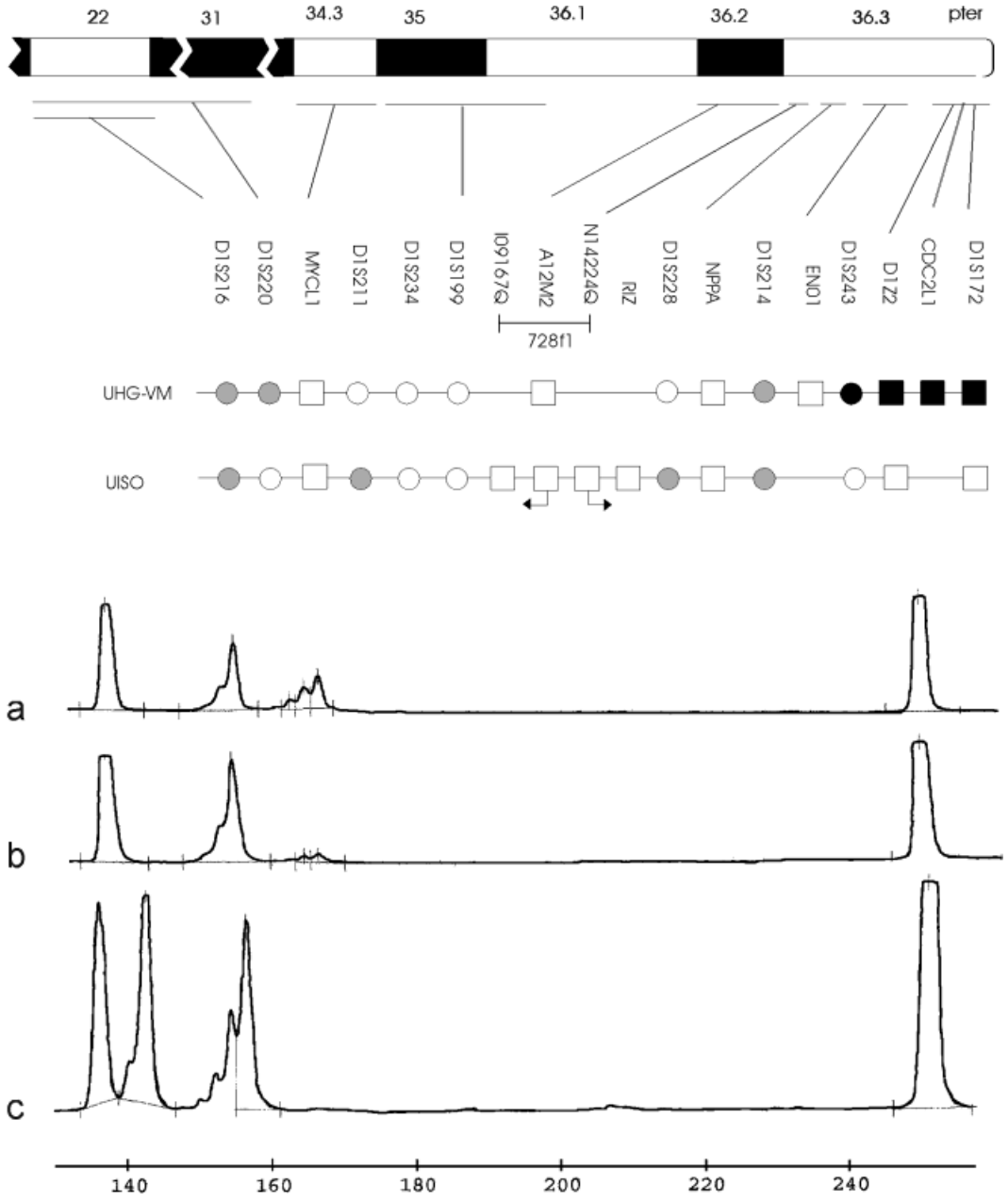

Figure 3. Top: Summary of the FISH and LOH data for chromosome arm 1p markers. Open square, presence of tested FISH markers on the der(1); solid square, absence of tested FISH markers on the der(1); open circle, heterozygous; solid circle, LOH; shaded circle, not informative for indicated microsatellite CA-repeat markers. Bottom: CA-repeat fragment analysis of microsatellite D1S243 in (**b**) MCC tumor UHG-VM, (**a**) peripheral blood of UHG-VM, and (**c**) MCC cell line UISO. Size range of this microsatellite is 142–170 bp. In every lane of the gel, an interval size marker was loaded (135 and 250 bp), flanking the microsatellite.

could play a similar role in the development of both tumors and possibly also in other neuroectodermal tumors that show 1p loss.

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2.1.2. Characteristic pattern of chromosomal gains and losses in Merkel cell carcinoma detected by comparative genomic hybridization

In view of the limited available genetic data for MCC, we decided to search for previously unrecognised genomic regions involved in MCC. To this purpose, we applied a technique called 'comparative genomic hybridization' (CGH) which allows screening of the entire tumor genome for gains, losses and gene amplification. In contrast with karyotyping, CGH does not require short-term cultivated tumor cells but can be performed with DNA isolated from fresh, frozen or paraffin-embedded material (Kallioniemi *et al.*, 1992; du Manoir *et al.*, 1993; du Manoir *et al.*, 1995). Characteristic patterns of gains and losses and/or amplification have been found previously in certain tumors or tumor subtypes (overviewed at http://www.progenetix.net). Recurrent losses may point at the chromosomal position of tumor suppressor genes involved in these tumors. Likewise, high level amplification often indicates the implication of particular proto-oncogenes. The identification of recurrent imbalances in MCC, could point at tumor suppressor genes or proto-oncogenes mapping in these regions and offer targets for further investigations.

In this study, 26 MCC tumors and eight MCC cell lines were analyzed by CGH. Overall frequent loss was observed for chromosomes 3p (46%), 5q (21%), 8p (21%), 10 (33%), 11q (17%), 13q (33%) and 17p (25%) and significant gains were observed for chromosomes 1 (63%), 3q (33%), 5p (38%), 8q (38%), 19 (63%) and X (41%). We found that this characteristic pattern of gains and losses in MCC resembled the CGH patterns previously observed for SCLC. This observation suggests that a number of common genes or pathways can be involved in the tumorigenesis of both MCC and SCLC. In contrast with SCLC, no high-level gene amplifications were observed in MCC.

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Characteristic Pattern of Chromosomal Gains and Losses in Merkel Cell Carcinoma Detected by Comparative Genomic Hybridization[1]

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ABSTRACT

Merkel cell carcinoma or small cell carcinoma of the skin is a rare skin cancer seen in increasing numbers in Queensland, Australia. In its clinical course and histopathology, it resembles small cell lung carcinoma (SCLC). Little is known of the genetic basis of this disease except for a number of cytogenetic studies and three loss of heterozygosity studies. Therefore, comparative genomic hybridization was performed to determine the characteristic DNA gains and losses that occur in this tumor. Comparative genomic hybridization analysis of 34 specimens from 24 patients revealed a pattern of gains and losses that closely resembles that seen in SCLC. Overall frequent loss was seen for chromosomes 3p (46%), 5q (21%), 8p (21%), 10 (33%), 11q (17%), 13q (33%), and 17p (25%). Significant gains were seen for chromosomes 1 (63%), 3q (33%), 5p (38%), 8q (38%), 19 (63%), and X (41%), with smaller numbers having gains for chromosomes 6, 7, 20, and 21. In contrast to SCLC, amplification in Merkel cell carcinoma is a rare event.

INTRODUCTION

MCC[3] is a neuroendocrine skin tumor with particularly aggressive clinical behavior, which appears to be increasing in incidence in Queensland, Australia. In 1992, it accounted for 4% of skin malignancies causing death in Queenslanders (1). Clinical behavior is similar to that of SCLC, with 25–30% of patients dying of their disease within 3 years (2, 3), attributable mainly to the propensity of MCC to metastasise early (50–80% of cases having regional lymph node involvement at presentation; Ref. 3). Histochemical markers are almost identical, making differential diagnosis of these diseases difficult where there is no obvious primary lesion. The majority of primary MCCs occur on the head and neck region or arms (3), implicating UV exposure in its etiology.

Cytogenetic investigations of MCC have not been common, with only 25 tumors and five cell lines reported in the literature (4–20).[4] So far, no recurrent chromosomal rearrangements have been identified that could be helpful in the identification of genes involved in MCC development; however, the short arm of chromosome 1 was frequently affected by inversions, translocations, and deletions. The observation of 1p36 abnormalities in two MCCs as the only karyotypic change suggested that genes located in this region play a role in MCC tumorigenesis (14).[4] Moreover, two distinct regions within 1p36 were recently shown to be implicated in MCC.[4] To date, only three large LOH studies on MCC have been reported, investigating the status of 1p, 3p, and 13q loci (21, 22).[5]

In view of the limited available genetic data for MCC, we decided to perform CGH on a series of tumors and tumor-derived cell lines from 24 patients. CGH allows screening of the entire genome for chromosomal gains, losses, and gene amplification. Therefore, it provides an opportunity to identify regions of the genome that undergo these types of genetic alterations in MCC and to increase our knowledge of this aggressive neoplasm.

MATERIALS AND METHODS

MCC Tumors and Cell Lines. In total, 34 DNA samples from 24 patients were analyzed. These include 26 tumors and eight MCC cell lines (Table 1). Clinical and LOH data for these tumors are summarized in Table 1. Seven cell lines were derived from tumors included in this study. Cell line UISO was described previously (18). Cytogenetic data of tumors and cell lines have been published (12, 23, 24) or will be reported elsewhere[6] (tumors UHG-VM and UHG-RM and cell lines MCC13, MCC14/2, MCC15, and MCC26). Within "Results" where patient loss or gain is referred to, only one tumor sample was counted for patients in which there were two samples examined and no cell line data were included.

CGH Analysis. Metaphase spreads were prepared from phytohemagglutinin-stimulated lymphocytes from healthy individuals according to standard procedures. Each batch of chromosome preparations was tested by hybridization (reverse painting) with DNA from the neuroblastoma cell line IMR32 with known DNA gains and losses (25) to assess the quality of the slides for CGH. Batches with poor hybridization (*e.g.*, strong staining of contours of sister chromatids and uneven or granular staining) were discarded. Slides were stored in plastic boxes with silica gel at −20°C before use. DNA was extracted from MCC cell lines and peripheral blood from a healthy male individual as described (26). Primary tumor DNA was extracted from 50-μm cryosections from biopsies frozen at −80°C. Labeling of DNAs, *in situ* hybridization, fluorescence microscopy, digital image acquisition, and processing were essentially done according to the methods of du Manoir *et al.* (27, 28) with minor modifications (25). 4′,6-Diamino-2-phenylindole images of metaphases were recorded before hybridization using a Leitz DM microscope, a high-sensitivity integrated monochrome charge-coupled device camera (Sony IMAC-CCD S30), and dedicated software (ISIS; MetaSystems GmbH, Altlussheim, Germany). Further processing of these images for CGH analysis was done using the ISIS CGH software (MetaSystems). For each case, 10–20 metaphases were analyzed. For evaluation of CGH data, average ratio profiles with fixed limits at 1.25 and 0.75 and SD limits (the width of the confidence interval being three times the SD) as well as individual ratio profiles were analyzed. A chromosomal region was considered to be over-represented (gain) or under-represented (loss) when the ratio profile crossed the SD limit. In tumors from female patients, a normal average ratio profile lies at or closely near the 1.25 limit, whereas a clear shift to the right of the average ratio profile *versus* the 1.25 limit was considered a gain. The reliability of the CGH procedure and software analysis was tested previously on well-characterized neuroblastoma cell lines. Particular attention was given in that study to the accuracy for detection of

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[3] The abbreviations used are: MCC, Merkel cell carcinoma; SCLC, small cell lung carcinoma; LOH, loss of heterozygosity; CGH, comparative genomic hybridization.

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Table 1 *Clinical and LOH data of tumors from 24 patients with MCC*

Tumor[a]	Sex	Specimen[b]	LOH[c] on 1p	LOH on 3p	LOH on 13	Nodes at first presentation	Survival[d] (months)
MCC1	F	Node	+	+	+	−	14
MCC2	M	Node	−	+	−	+	13
MCC3	M	Node	+	+	+	+	23 *DA
MCC4	M	Node	−	−	+	−	40
MCC5	F	Rec	+	+	+	−	26
MCC7T1	F	Rec	+	+	+	−	42
MCC7T2	F	Met	+	+	+	−	42
MCC8	M	Rec	+	−	+	+	16
MCC9	M	Rec	−	+	+	−	9 I/C
MCC11	M	Rec	+	+	+	−	12 I/C
MCC12	M	Rec	−	−	+	−	9
MCC13	F	Rec	−	−	+	+	24 *DA
MCC14T1	M	Node	−	+	+	−	5
MCC14T2	M	Node	ND	ND	ND	−	5
MCC15	M	Rec	+	−	−	−	11
MCC16	M	Rec	+	−	−	−	17
MCC17	M	Rec	+	+	+	−	12
MCC18	M	Rec	+	+	+	−	42 *DP
MCC20	F	Node	ND	+	ND	+	49 *DA
MCC21	M	Node	−	−	−	−	33 *DP
MCC22	M	Node	+	+	−	+	24 *DP
MCC24	M	Rec	+	−	+	−	17
MCC26T1	F	Rec	+	+	−	−	9
MCC26T2	F	Node	+	+	−	−	9
UHG-VM	F	Met	+	ND	ND	+	4
UHG-RM	F	Met	−	ND	ND	+	22
UISO	F	Prim	−	ND	ND	?	?

[a] Cell line MCC26TC is derived from MCC26T1; cell lines MCC14/1TC and MCC14/2TC are derived from MCC14T1.
[b] Rec, recurrence at site; Node, specimen taken from regional lymph node; Met, metastasis; Prim, primary tumor.
[c] +, LOH detected within region of study; −, no LOH detected; ND, not done.
[d] Survival is given in months, where * after number the patient is still alive with disease (DP) or without disease (DA). In two cases the patient died from other causes (I/C).

small distal 1p deletions and *MYCN* amplification (25). As a control, normal-to-normal hybridization was performed.

RESULTS

The results of CGH analysis of 34 samples of tumor and tumor cell line DNA from a total of 24 patients are summarized in Fig. 1. Full lines in Fig. 1 represent over-representation (right) and under-representation (left) as calculated by the CGH software using statistical analysis. *Dashed lines* were used if individual profiles showed a consistent shift toward the lower or upper threshold. High-copy number amplification was notable as a large shift of the ratio profile for a chromosomal subregion and was represented as *bold lines*. *Open boxes* were used for unusual high over-representation of large chromosomal regions. Selected examples of average ratio profiles with over- and under-representations are shown in Fig. 2.

Chromosomal imbalances were detected in all analyzed DNA samples. The average number of imbalances was 9, with the highest score of 19 imbalances observed in MCC26T1.

DNA Losses. DNA under-representation was most frequently seen for the short arm of chromosome 3 in 16 of 34 specimens (47%) representing 11 patients (46%) (Figs. 1 and 2*g*). In two specimens (both from MCC26), the entire chromosome 3 was under-represented, but in most other cases 3p under-representation coincided with 3q over-representation. In two patients (MCC7 and MCC14), partial deletions of 3p were observed (*e.g.*, MCC7T1; Fig. 2*h*). The smallest deletion was found in tumors MCC14T1 and MCC14T2 and cell line MCC14/1TC.

Other frequently under-represented regions were chromosome 5q [6 of 34 specimens (18%) from five patients (21%)], chromosome 8p [seven specimens from five patients (both 21%)], chromosome 10 [nine specimens (26.5%) from eight patients (33%)], chromosome 11q [six specimens (18%) from four patients (17%)], chromosome 13q [nine specimens (26.5%) from eight patients (33%)], and chromosome 17p [eight specimens (24%) from six patients (25%)].

It should be noted that some DNA losses may actually be scored as DNA gains for the remaining part of the chromosome. This is illustrated by the chromosome 13 profiles for MCC5T and MCC5TC (Fig. 1). MCC5T shows under-representation of the proximal part of 13q, whereas in MCC5TC, over-representation of the distal part of 13q is observed. The MCC5TC profile could be explained by the presence of extra copies of the derivative chromosome 13 or translocated distal 13q material in this cell line. A similar phenomenon was seen for deletions of 1p in neuroblastomas and can be explained easily by increase of the copy number of the deleted chromosome (25, 29).

The smallest chromosome 13q deletion was observed in MCC11T (Fig. 2*j*), which defines a consensus deletion at 13q14→q21.

Under-representation for a small distal segment of 2q (2q37→qter) was observed in MCC7T1 and MCC7T2. In both tumor biopsies MCC26T1 and MCC26T2 (taken 3 months apart) and the derived cell line MCC26TC, a shift (statistically not significant) in the average ratio profile for chromosome 2 (2p13→p16) toward the lower threshold was observed (Fig. 2*b–d*). The consistent but statistically not significant deviation of the chromosome 2 average ratio profile can be explained both by the small size of the deletion and by the presence of three chromosomes 2, as revealed by karyotypic analysis (data not shown). Also, the presence of a second clone of cells without the interstitial deletion cannot be excluded.

DNA Gains. Gains for chromosome 1 (either whole arm or the entire chromosome) were most frequently observed, with 19 (56%) specimens representing 15 (63%) patients. For nine (26%) specimens, this represented a gain of the whole of the chromosome. For the short arm, 14 specimens (42%) from 13 patients (54%) had a gain of material, and for the long arm, 14 specimens from 10 patients (42%) showed a gain. Chromosome 19 was also over-represented in the majority of patients for both the p and q arms, with 15 specimens (44%) from 12 (50%) patients having a gain of 19p and 19 (56%) specimens from 15 (63%) patients having a gain of material of the q arm. Other frequent gains included 3q [13 specimens (38%), eight patients (33%)], 5p [15 specimens (44%), nine patients (38%)], 8q [13

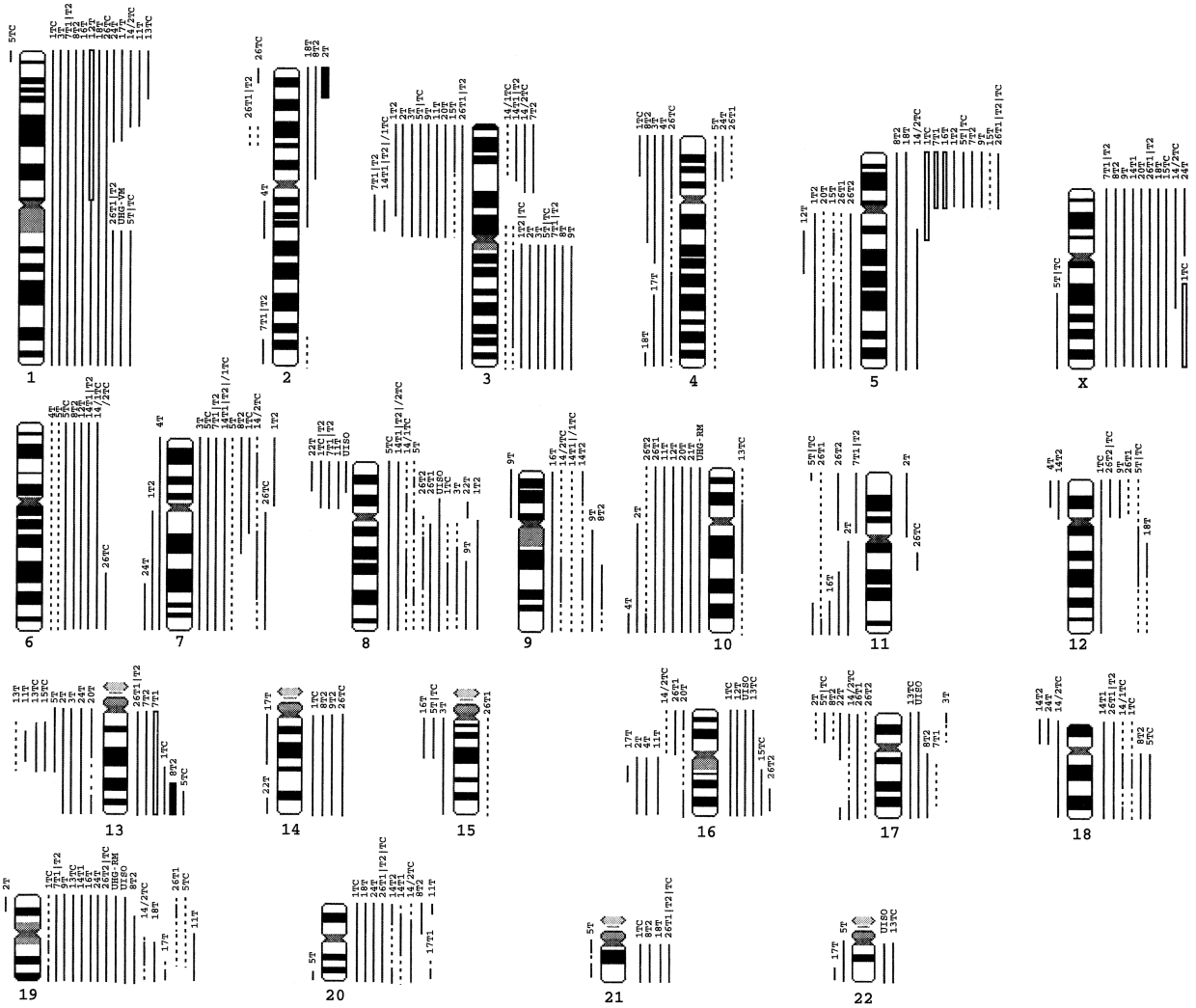

Fig. 1. Ideogram of DNA copy number changes in 34 samples of MCC tumor (*T, UHG-VM,* and *UHG-RM*) and tumor cell line (*TC* and *UISO*) DNA from a total of 24 patients. *Bars* on the *left* indicate copy number decreases; *bars* on the *right* show copy number increases; *open boxes* represent multiple copies of large chromosomal regions; *bold bars* represent high-copy number amplification. *Dotted lines* indicate consistent over- or under-representation but within the confidence limits.

specimens (38%), nine patients (38%)], and X [14 specimens (41%), 10 patients (42%)]. Gains in a smaller number of tumors and cell lines were noted for chromosomes 6, 7, 13q, 18q, 20, and 21. In MCC12T, the average ratio profile suggests the presence of multiple copies of 1p. Similar abnormal profiles for 5p were observed in MCC1TC (Fig. 2*i*), MCC7T1, and MCC16T.

Gene Amplification. Gene amplification was found in only two samples. For MCC8T2, the CGH analysis revealed a high-level copy number for the distal region of 13q, 13q31→qter (Fig. 2, *k* and *l*). Likewise, an amplification at 2p23-24 was observed in MCC2T (Fig. 2, *e* and *f*). Both amplifications were also noted after reverse painting of tumor DNA to normal metaphases (data not shown).

Multiple Alterations. Analysis of the data demonstrated seven specimens (21%; MCC1TC, MCC5TC, MCC7T1, MCC7T2, MCC8T2, MCC26T1, and MCC26T2) from five patients (21%) that showed a combination of imbalances for chromosome 3 and gains for 1q, 5p, 19q, and 13q.

Correlation of Number of Gains and Losses *versus* Survival and Aggressiveness. Prognosis is generally poor in MCC; however, an attempt was made to correlate outcome with genetic change. The patient series was divided into two groups: those who survived for >24 months (group 1) and those who survived for <24 months after diagnosis (group 2). Survival data are included in Table 1. For each group, the total number of imbalances was divided by the number of patients, giving an average number of imbalances per tumor for each group. The average numbers of imbalances were 6.6 for group 1 (n = 9) and 11.2 for group 2 (n = 11); however a Student's t test revealed that these were not significantly different (P = 0.288, 8 degrees of freedom).

DISCUSSION

We have performed CGH analysis for genome-wide screening for losses and gains of DNA sequences in a large series of MCC tumors

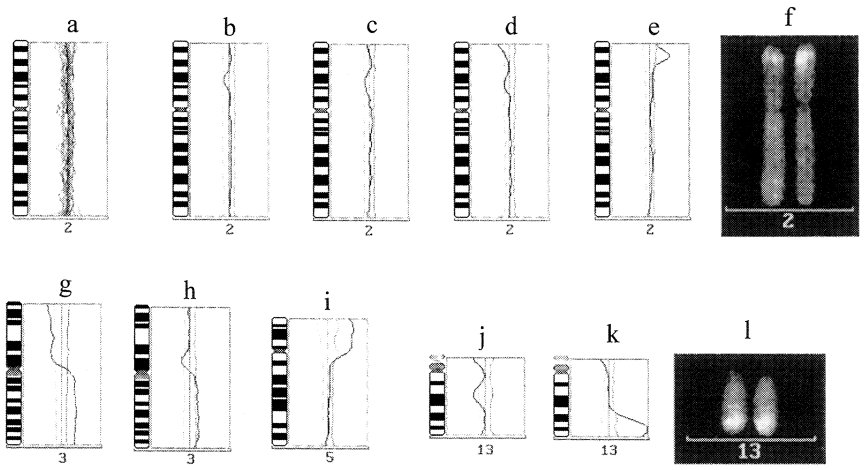

Fig. 2. Examples of average ratio profiles. *a*, Normal ratio profiles of 15 individual chromosomes 2 of MCC11T showing limited fluctuations in the green to red ratio; *b–d*, average ratio profiles of chromosomes 2 for MCC26T1, MCC26T2, and MCC26TC, respectively, showing a consistent but statistically not significant deviation of the average ratio profile at 2p13→p16; *e*, average ratio profiles of chromosomes 2 for MCCT2 demonstrating high-copy number amplification at 2p23–24; *f*, CGH image of two chromosomes 2 of MCCT2, the strong *white* staining corresponds to the increased fluorescence intensity of the tumor DNA detected by FITC; *g* and *h*, average ratio profiles of MCC5TC and MCC7T1 of chromosomes 3 showing an entire 3p under-representation and 3q over-representation and a small 3p11→p14 deletion, respectively; *i*, average ratio profiles of MCC1TC for chromosomes 5 showing multiple copies of 5p; *j*, average ratio profiles of MCC11T for chromosomes 13 demonstrating a partial 13q14→21 deletion; *k* and *l*, average ratio profiles and CGH image of chromosomes 13 of MCC8T2 demonstrating a high-copy number gene amplification at 13q31→qter.

and MCC cell lines derived from some of these tumors. The available data on genetic alterations occurring in MCC are sparse. Cytogenetic analysis revealed preferential involvement of chromosome 1 and loss of distal 1p material (4, 6, 7, 10–14, 17, 18, 20).[4] LOH studies demonstrated a high incidence of allelic loss for 1p, 3p and 13q markers (21, 22).[5] We have shown previously that particular gains and losses of chromosomal regions that were not noted on karyotypic analysis could be detected by CGH analysis (25), and CGH analysis is particularly suited for scanning tumor genomes for other regions preferentially lost during tumorigenesis and not identified so far by karyotype or LOH analyses.

The present study on MCC reveals a characteristic pattern of genomic imbalances, including over-representation of chromosomes 1, 3q, 5p, 6, 7, 8q, 19, 20, and 21. Frequent loss of DNA sequences was observed for 3p, 5q, 8p, 10, 11q, and 17p. For chromosome 13, both losses and gains of DNA copy number were observed. Similar patterns of chromosomal imbalances have been observed in SCLC (30–33) and head and neck squamous cell carcinomas (34–36). As in these tumors, simultaneous gain of one arm of a chromosome and loss of the other arm for chromosomes 3, 5, and 8 were also observed in MCC. These CGH profiles could be explained by formation of isochromosomes and may correlate to a low-level amplification of many genes, which might induce a growth advantage for cells carrying a 3q, 5p, or 8q gain.

In fact, the occurrence of i(3q), i(5p), and i(8q) has been reported in SCLC (37) and head and neck squamous cell carcinomas (38). In cytogenetic studies of MCC, i(5p) and i(8q) have been reported sporadically (5, 6, 20), but an i(3q) has not been described. A possible reason for the discrepancy between CGH and cytogenetic findings could be the presence of other structural aberrations that lead to similar ratio profiles.

DNA under-representation for chromosome 3p most frequently encompassed the entire short arm. CGH findings were in agreement with LOH data for 18 of 22 investigated patients. A consensus deletion region was defined as 3p11→p14 by CGH compared with 3p13→p21.1 (21), suggesting that the critical region is at 3p13–14. CGH analysis of SCLC revealed a similar consensus region for 3p DNA loss (33). In this same study, a second more distal region of loss, 3p21→pter, was identified. In the present study, no small deletions encompassing 3p21→pter were observed. However, this region was lost in 38% of tumors because of loss of an entire 3p arm; thus, implication of a distal tumor suppressor gene cannot be excluded. Deletions of 3p have been observed in tumors of many organ systems, including kidney, ovary, head and neck, and testis (39), thus strongly suggesting that this chromosomal region harbors one or more tumor suppressor genes implicated in these tumors. A number of tumor suppressor genes on 3p have been identified, including the *von Hippel-Lindau* gene (*VHL*; Ref. 40), the *β-catenin* gene (*CTNNB1*; Ref. 41), and the *FHIT* gene (42). Of these three, the *FHIT* gene is of particular importance, because it maps within the SCLC and MCC consensus region on 3p14.2. The *FHIT* gene was shown to exhibit exonic loss in 80% of SCLCs, 40% of non-small cell lung cancers (43), and 57% of MCCs (44), thus suggesting an important role for this gene in lung and Merkel cell carcinogenesis.

DNA loss on chromosome 13q was found in nine (26.5%) MCC tumors and cell lines. CGH findings were in agreement with LOH data for 10 of 22 investigated patients. In three tumors and two cell lines, partial 13q deletion occurred, defining a consensus region to 13q14→21, a region that encompasses the *RB1* locus at 13q14. This is in agreement with the observation of LOH for 13q in 75% of investigated MCCs and is consistent with the finding that a number of MCC cell lines do not contain detectable levels of RB1 protein (22). Deletions of 13q have been reported in various other neoplasms, including retinoblastoma, SCLC, prostate cancer, renal cancer, and B-cell lymphocytic leukemia (39). In SCLC, DNA copy number losses for 13q, including the *RB1* locus, were found in 86% of the cases (33). It remains to be determined whether the *RB1* gene is the only gene in this region that plays a role in MCC tumor biology.

Loss of an entire chromosome 10 was observed in 21% of cases. In one tumor, under-representation was restricted to the 10q arm. Deletions of chromosome 10q have been described in prostate (45), renal (46), and endometrial (47) cancer, SCLC (33), melanoma (48), meningioma, and glioblastoma multiforme (49). Recently, a strong candidate tumor suppressor gene at chromosome 10q23.3 was identified. The gene was designated *MMAC1*, for mutated in multiple advanced cancers 1, because mutations in this gene were found in glioma, prostate, kidney, and breast carcinoma tumors or cell lines (49). This gene maps in the region of the susceptibility gene for Cowden disease, an autosomally dominant multiple cancer syndrome in which patients present with hamartomas of thyroid, breast, skin, and gastrointestinal tract. This has been mapped to a 6-cM interval on chromosome 10q22-23 (50).

Chromosomal region 8p was reported to be lost frequently in SCLC (33), prostate (51), bladder (52), renal (53), colorectal (54), hepatocellular (55), and male (56) and female (57) breast cancer and was also found to be under-represented in 21% of MCCs investigated in this study. LOH studies have delineated at least two regions on 8p around 8p21 and 8p22, which could harbor tumor suppressor genes (57).

Under-representation of 5q was observed in 18% of all specimens. In all except one case, the entire long arm was involved, probably as a result of isochromosome formation. The smaller deletion, seen in MCC12T, encompasses 5q12→21, a segment that could contain the *MCC* and *APC* loci, which have been mapped to 5q21→22. Further analysis is required to verify the absence or presence of these loci in this tumor. In SCLC, two distinct regions of loss on 5q were identified, 5q21→22 and 5q31→qter (33), the first of which could coincide with the deleted region on chromosome 5 in tumor MCC12T.

Other chromosomal regions known to harbor tumor suppressor genes that showed DNA loss were 11p (*WT1*), 16q (*cadherin* gene family members), and 17p (*TP53*), each of which forms targets for further investigation in MCC.

The frequently observed rearrangements of chromosome 1 and deletions of 1p by cytogenetic and LOH analysis are not apparent from the CGH profiles. Possible explanations for the discrepancies between CGH *versus* cytogenetic and LOH data are that: (*a*) some deletions may be small and telomeric and thus not detectable by CGH; (*b*) a number of imbalances detected as over-representations may in fact represent extra copies of a chromosome with a deletion, *e.g.*, over-representation of 1q as a result of extra copies of a chromosome 1 with a deletion of the short arm; (*c*) in MCC, other structural rearrangements than deletions may affect genes on 1p, such as the 1;5 translocation in MCCT2 (see below), because balanced translocations cannot be detected by CGH; and (*d*) maternal and paternal chromosomes 1 are not present in equal copies, resulting in allelic imbalances rather than LOH (see below).

High-copy number amplifications for chromosomal subregions were found in only two MCC tumors and thus are a rare event in MCC. In one tumor (MCCT2), the amplified sequences mapped to 2p23-24, a region containing the *MYCN* locus. *MYCN* amplification is a frequent observation in advanced neuroblastomas (58). These *MYCN*-amplified neuroblastomas almost invariably carry 1p deletions, and it has been suggested that a so-called "suppressor of *MYCN*" gene is deleted in these tumors (59). In this respect, the finding in this tumor of an apparently balanced 1;5 translocation with a breakpoint in 1p36 (12) may be of relevance, because the same gene involved in *MYCN*-amplified neuroblastomas may be implicated in MCC, albeit rarely. Further analysis of the 1p36 breakpoint in this tumor could thus lead to more detailed localization or cloning of this putative suppressor of the *MYCN* gene.

The second high amplification was found for 13q31→qter in tumor MCC8T2. Similar amplification was described previously in SCLC (31, 33), head and neck squamous cell carcinoma (36), and glioma and glioblastoma multiforme (60). A possible candidate gene implicated in this amplification region is *RAP2*, which encodes a Ras-related protein and is located at 13q34 (61).

Gains of several chromosomal regions were detected. Extra copies of chromosome 1 were observed in 33% of patients. Over-representation of 1p material was present in an additional 21% of patients. This finding is in agreement with the CGH data and interphase analysis on three MCC tumors (62).

Copy number increase on 3q was observed in 35% of specimens. As mentioned above, a similar gain of 3q, often in association with 3p loss, has been described in SCLC and head and neck squamous cell carcinomas. Extra 3q material has also been reported in other tumor types, such as brain, bladder, ovarian, and cervical cancer (39). In head and neck squamous cell carcinomas, a minimal common region at 3q26-q27 was defined (35, 36). This region is known to contain certain proto-oncogenes such as *LAZ3* and *BCL6*.

Over-representation of 8q was observed in 38% of specimens. The smallest region involved was 8q22→qter. Gain of 8q was also observed in uveal melanoma (35, 63), SCLC (30–33), head and neck squamous cell carcinomas (36), hepatocellular carcinoma (55), and prostate cancer (64–66). In the latter, extra 8q was typically found in recurrent tumors, suggesting that a gene on 8q could be involved in progression of prostate cancer. The *MYCC* oncogene on 8q24 is known to be amplified in subsets of SCLC and was shown to be overexpressed in uveal melanoma (67). However, this oncogene is unlikely to play a major role in MCC, because none of the 24 tumors showed amplification in this region.

Chromosome 19 was over-represented, with the majority of tumors showing a gain for both chromosome arms: 44% for the p arm and 50% for the long arm. In the 19p13 region, there appears to be a number of genes that undergo translocation events in acute lymphoid and acute myeloid leukemias, which may have an oncogenic phenotype. One of these, *EEN*, is an α-helical protein with Src homology in the COOH-terminal end (68).

There appear to be a number of common genetic changes in MCCs and SCLCs, which may lead us to identify genes important for maintenance of the neuroendocrine cell phenotype and for tumorigenic alteration of Merkel and pulmonary neuroendocrine cells.

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2.1.3. Combined karyotyping, CGH and M-FISH analysis allows detailed characterization of unidentified chromosomal rearrangements in Merkel cell carcinoma

In addition to CGH, we applied M-FISH to identify and characterize chromosomal rearrangements occurring in MCC in further detail (Speicher *et al.*, 1996). M-FISH allows detection of balanced translocations which is not possible with CGH. In complex karyotypes or in poorly spread and banded metaphases a significant number of rearrangements can be overlooked by banding analysis only, but may be detected by M-FISH. In contrast with conventional FISH, prior knowledge of the nature of chromosomal rearrangements is not required. Previous studies illustrated that an optimal resolving power was obtained for M-FISH when combined with karyotyping, CGH and standard FISH (Van Roy *et al.*, 2001; Van Limbergen *et al.*, 2002).

In this report, six MCC cell lines and one metastatic MCC tumor were analyzed by karyotyping, CGH, M-FISH and FISH with region specific probes. Almost all studied cases showed near-diploid karyotypes with simple karyotypic changes, multi-way translocations or various numerical and structural changes. The major chromosomal changes observed were chromosomal rearrangements involving the short arm of chromosome 1 and the long arm of chromosome 3, gain of 5p material and loss of chromosome 10. In addition, M-FISH allowed identification of many previously unnoticed rearrangements distributed throughout the whole genome. Our accurate cytogenetic profiling of the chromosomal abnormalities occurring in MCC opens new perspectives in the search for candidate genes located in these chromosomal regions.

Combined karyotyping, CGH and M-FISH analysis allows detailed characterization of unidentified chromosomal rearrangements in Merkel cell carcinoma

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Abbreviations: MCC: Merkel cell carcinoma, CGH: comparative genomic hybridization, M-FISH: multiplex-fluorescence in situ hybridization

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Abstract

Merkel cell carcinoma (MCC) is a rare aggressive neuroendocrine tumor of the skin. Cytogenetic studies indicated that deletions and unbalanced translocations involving chromosome 1 short arm material occur in 40% of the investigated cases. Recurrent chromosomal imbalances detected by CGH analysis were loss of 3p, 10q, 13q and 17p and gains of 1q, 3q, 5p and 8q. In order to study genomic aberrations occurring in MCC in further detail we combined karyotyping, CGH and M-FISH, a strategy which proved to be successful in the analysis of other malignancies. Analysis of 6 MCC cell lines and one MCC tumor revealed mostly near-diploid karyotypes with an average of 5 chromosomal rearrangements. The observed karyotypic changes were heterogeneous with 3 to 27 breakpoints per case leading to imbalance of the involved chromosomal regions confirmed by CGH. Chromosomal rearrangements involving the short arm of chromosome 1, the long arm of chromosome 3 and gain of 5p material were the most frequently observed abnormalities in this study. In keeping with previous observations, this series of MCCs showed no evidence for high level amplification. We provided a detailed description of chromosomal translocations occurring in MCC which could be useful to direct future intensive investigation of these chromosomal regions.

Introduction

Merkel cell carcinoma (MCC) is a rare aggressive tumor which occurs mainly on sun-exposed areas of the skin in elderly people. The tumor is thought to originate from Merkel cells. These cells are located in the basal layers of the epidermis and are the mechanoreceptors of the skin.[1]

Up to now, cytogenetic analysis of 27 tumors and 4 MCC cell lines have been reported.[2-20] Recurrent reciprocal translocations leading to fusion genes or activation of dominantly acting oncogenes have thus far not been identified in this tumor. Structural abnormalities involving the short arm of chromosome 1 were observed in 40% of the studied cases often leading to loss of distal 1p-material, which suggests the involvement of one or more tumor suppressor genes located in this region. Detailed molecular cytogenetic analysis of two chromosomal 1p-rearrangements revealed that at least two distinct critical regions within 1p36 were involved in MCC.[20] Loss of heterozygosity (LOH) studies for 1p-markers confirmed the high incidence of allelic imbalance for the 1p region and showed complex patterns of losses for the distal part of chromosome 1.[21-23] High incidence of 1p losses has also been described in many other tumors including colon and breast carcinomas. Several candidate tumor suppressor genes have been mapped to the various shortest regions of overlap on 1p but none of them seem to be directly involved in tumors with 1p loss.[24] Comparative genomic hybridization (CGH) on 26 MCC tumors and eight cell lines showed recurrent gains of chromosome 1q, 3q, 5p, 8q, 19 and X and losses of 3p, 5q, 8p, 10q, 11q, 13q and 17p.[25] In addition, losses of 3p, 9p, 10q and 13q were evidenced by LOH analyses.[26-29]

In view of our limited understanding of the underlying genetic defects leading to MCC and given the extremely poor response of MCC to current treatment protocols, further refined analysis of chromosomal changes occurring in MCC is warranted. In the present study we combined karyotyping, CGH, FISH with region specific probes and multiplex-fluorescence in situ hybridization (M-FISH)[30] to obtain a detailed description of the chromosomal aberrations in 6 MCC cell lines, 3 of which were not previously subjected to cytogenetic investigation, and one new MCC tumor. The findings in these cell lines and tumor are discussed and compared to the available cytogenetic data for MCC.

Material and Methods

MCC cell lines and MCC tumor sample

Details of morphology, DNA content and immunohistochemistry of cell line MCC13 was previously described by Leonard *et al*.[31]. Characterization and cytogenetic data of cell line UISO were published by Ronan *et al*.[12]. Cytogenetic and morphologic analysis of cell line MKL-1 and its two subclones was described by Rosen *et al*.[4]. Only the two subclones of MKL-1 were analyzed in this study. Cell line MCC26 was established at the Queensland Radium Institute Laboratory, Queensland Institute of Medical Research, Brisbane, Queensland, Australia and cell line MKL-2 at the Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL, USA. The latter was established from a 72-year-old white male of which the pathological records were unfortunately not available.

Fresh tumor material from a patient with Merkel cell carcinoma (case UHG-RM) was collected at the Department of Medical Oncology, Ghent, Belgium and primary cell cultures were established at the Center for Medical Genetics according to standard procedures.

Cytogenetic analysis

Cell culture, harvesting, slide preparation and G-banding were performed according to standard procedures. Karyotypes were described according to the ISCN[32] guidelines.

Comparative genomic hybridization

CGH data were published for cell lines UISO, MCC13, MCC26 and MCC tumor UHG-RM.[25] In this study, additional CGH analysis was performed for MKL-1 subclone 2. Labeling of DNA, in situ hybridization, fluorescence microscopy, digital image acquisition and processing were done according to du Manoir *et al*.[33,34], with minor modifications.[35] 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Roche Molecular Biochemicals, Brussels, Belgium) images of metaphases were recorded prior to hybridization using a fluorescence microscope, a high-sensitivity integrated monochrome CCD camera (Sony IMAC-CCD S30), and dedicated software (ISIS, MetaSystems, Altlussheim, Germany). Further processing of these images for CGH analysis was performed with the ISIS CGH software (MetaSystems). For each case, ten to twenty metaphase cells were analyzed. For evaluation of CGH data, average ratio profiles with fixed limits at 1.25 and 0.75 and standard deviation limits (the width of the confidence interval being three times the standard deviation) as well as individual ratio profiles were analyzed. A chromosomal region was considered to be overrepresented (gain) or under-represented (loss) if the average ratio profile crossed the standard deviation limit. As a control, normal to normal hybridizations were performed.

M-FISH analysis

M-FISH for differential staining of all human chromosomes was performed according to Van Roy *et al*.[36]. Briefly, the '24*X*Cyte' probe kit was purchased from MetaSystems (Altlussheim, Germany), and contains combinatorially labeled chromosome paints obtained from degenerated oligonucleotide primer polymerase chain reaction (DOP-PCR) amplified microdissected chromosomes. Fluorescein isothiocyanate (FITC), SpectrumOrange (SO), TexasRed (TR) and diethylcoumarine (DEAC) were used for direct detection whereas biotin was indirectly visualized with streptavidinCy5. Metaphase slides

were pre-treated with RNase and pepsin. Slides were denatured with 70% formamide/2xSSCP at 80°C for 5 min. Probe mix was denatured at 75°C for 5 min, incubated at 37°C for 30 min and subsequently applied to the slides under a 18x18 mm coverslip. After 2-4 days of hybridization, slides were washed with 50% formamide/2xSSC (pH 7.3-7.5) at 42°C (3x5 min), followed by three washes in 2xSSC (42°C). Biotin-labeled probes were detected with streptavidinCy5. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing DAPI (Roche Molecular Biochemicals, Brussels, Belgium) counterstain. Fluorescent images were captured with a Zeiss axioplan epifluorescence microscope (Carl Zeiss) (8 position filter wheel) equipped with a CCD camera. All six fluorochromes including the DAPI counterstain were captured sequentially, using single-bandpass filters. Color composite images were constructed from the separate monochrome images and processed using the isis/mFISH imaging system (MetaSystems, Altlussheim, Germany). Distinct pseudo colors were assigned to each pair of homologous chromosomes and to the X and Y chromosome based upon the fluorescent color signature for each pixel. At least five metaphases from each case were analyzed by M-FISH.

FISH with whole chromosome paints and region specific probes

Subsequent to M-FISH analysis, additional dual color FISH with whole chromosome plasmid libraries[37] and region specific probes was performed for confirmation or further characterization of chromosomal rearrangements. Hybridization and detection were done according to Van Roy *et al*.[38]. Refined localization for chromosome 1p breakpoints was performed using the following probes: pUC1.77 (D1Z1, for the heterochromatic region of chromosome 1)[39], p1-79 (D1Z2, 1p36.33)[40], p73 cosmid (1p36.33)[41], c176g8 (*DFFB*, 1p36.3)[42], 12A-2 (*NPPA*, 1p36.31-p36.23)[43], cHE2.6 (A12M2, 1p36.1)[44], C1-heir1D (*ID-3*, 1p36.2-p36.1)[45], cosmid probe ICRFc112B2137Q (*EXTL1*, 1p36.1)[46], P1294 (*Connexin27*, 1p35.1)[47], YAC clone 209E9 (*MYCL1*, 1p34.3)[48] and YAC clone 958H24 (1q32-q41).

Additional locus specific probes used in this study were: LSI D7S522/Cep7 (7q31/cen7), LSI13 (13q14), LSI CCND1 (11q13), LSI 5qEGR1/D5S23 (5q31/5p15.2), Telvys 14q, Telvys 17q, Telvys 18q, dual color probes LSI ABL/BCR (9q34/22q11), LSI inv(16) (16q22tel/16q22cen), LSI MLL (spanning the *MLL* gene, 11q23tel/11q23cen), LSI DiGeorge/LSI ARSA (22q11.2/22q13), LSI SRY/CEP X (Yp11.3/Xp11.1-q11.1) (Vysis, Downers Grove, IL). BAC clones RPCI-11 178P16 (16q23-24), RPCI-11 1094H24 (17q21), *EVI1* BAC clones RP11-33A1, RP11-627P8, RP11-3J21, RP11-48N2 and RP11-816J6 (3q26) (Children's Hospital Oakland Research Institute (C.H.O.R.I.) in Oakland, California, USA), PAC clones RPCI-1 32H9 (17q11), RPCI-1 1094H24 (17q21), RPCI-1 300F16 (*RBP1*, 3q23)[49] and RPCI-1 83I4 (*SHOX2*, 3q25-q26.1)[50] (Ressourcenzentrum Primärdatenbank in the Max Planck-Institut für Molekulare Genetik in Germany (RZPD))[51], centromeric probes pα3.5 (cen3)[52], p17H8 (cen17)[53], pI90.22 (cen22)[54] and Y97 (cenY)[55], p82H (all centromeres)[56], pHUR98 (for the heterochromatic region of chromosome 9)[57], pHUR195 (for the heterochromatic region of chromosome 16)[57] and D1S80 (this probe was originally mapped to 1p[58] but hybridized to the short arms of acrocentric chromosomes (personal findings)).

Case reports

Case 1 (cell line MCC26)

MCC26 was established from the recurrence of a tumor from a 82-year-old woman prior to radiotherapy. The patient was initially referred to the Queensland Radium Institute (Brisbane, Australia) with a lesion to the left lateral calf. This lesion was confirmed by biopsy to be an MCC. Immunohistochemical analysis showed positivity for neuron specific enolase (NSE) and low-molecular-weight keratin and negativity for S100 and high-molecular-weight keratin. After wider excision the tumor recurred near the primary site and radiotherapy was started. Three months later the patient presented with lymphadenopathy in the left groin, where examination showed several nodes up to 4 cm in diameter. The patient received palliative radiotherapy at 20 Gy in 5 fractions over 1 week. The patient died three months later.

Immunohistochemistry of the MCC26 cell line at early passage showed a positive reactivity for NSE and negative staining for synaptophysin, GFAP, NFP, S100, CAM 5.2, LCA, chromogranin and somatostatin.

Case 2 (tumor UHG-RM)

A 62-year-old woman was referred to the hospital because of an induration on the left thigh, steadily growing over a 5 week period. Physical examination revealed a tumoral mass on the left thigh, painful by palpation, with a reddish overlying skin. There was an enlarged lymph node with a diameter of 3 cm in the left inguinal region. Blood counts and biochemistry were completely normal, as was the serum level of calcitonin. A complete resection of the tumor and an inguinal lymph node dissection was done, followed by radiotherapy at the tumorbed and the left groin.

Histopathologically, the tumor consisted of nodular aggregates of small monoform atypical cells with a fine chromatin network. Multiple mitoses were seen. The tumor was infiltrated by lymphocytes. The inguinal lymph nodes were infiltrated by the above described malignant cells. Immunochemistry for NSE, CAM 5.2, EMA and chromogranin A was positive whereas S100 and LCA were negative. These findings correlated with a neuroendocrine tumor of the skin or Merkel cell carcinoma.

Results

Karyotypes described by banding analysis only and revised karyotypes following FISH analysis are listed in Table 1. Composite karyotypes were described for cell lines exhibiting karyotypic heterogeneity. M-FISH analysis in combination with the available cytogenetic and CGH data led to an improved karyotype description for all analyzed MCC cell lines and the metastatic MCC tumor. All structural aberrations found with M-FISH could be confirmed using conventional FISH.

Six of the seven studied cases (MCC13, MKL-1 (subclone 1 and 2), MKL-2, UISO and tumor UHG-RM) were near-diploid whereas MCC26 showed a hypotriploid karyotype. Except for the MKL-1 subclones, all cases had a complex karyotype (more than 3 structural chromosomal changes) often showing unbalanced rearrangements leading to deletions and gains, usually in combination with numerical abnormalities. Cytogenetic analysis of 6 cell lines and one MCC tumor revealed a total of 23 partially defined chromosome aberrations and 7 markers which could all be further characterized by M-FISH analysis. Gene amplifications were not detected. M-FISH images of complete karyotypes or selected chromosomal rearrangements are shown in Fig. 1. The chromosomes most frequently involved in rearrangements were in order of frequency 3, 1, 5, 7, 13, 14, 8, 9, 17 and 22. Chromosomal arms most commonly affected were the long arm of chromosome 3 and the short arm of chromosome 1. Chromosomal breakpoints determined by M-FISH, banding analysis and dual-color FISH and imbalances detected by CGH are shown in Fig. 2. Rearrangements at 3q23, 3q25-q26.1 and 9q34 were each seen in 2 different cases.

In MCC13, M-FISH revealed a three-way translocation involving the chromosomes 7, 8 and 14 (Table 1, Fig. 1a). This rearrangement was apparently balanced, as CGH revealed no gains or losses for these chromosomes. In addition, an add(9)(q34) and a small marker chromosome were characterized as a der(9)(9pter→q34::13q22.2→13qter) and a der(13)(13pter→13q12.3::16q23q24→16qter) respectively (Table 1, Fig. 1a). Loss of 13q12.3→q22.2 material was confirmed by CGH (data not shown). Karyotypic analysis of MCC13 also showed a del(12)(q12q14) in half of the analyzed mitoses which was not detected by M-FISH nor CGH.

In MCC26, M-FISH revealed complex translocations involving the chromosomes 1, 3, 4 and 7. Other complex rearrangements involved chromosomes X, 8, 14, 16 and 21 which could be partially described in further detail through CGH analysis (Table 1). In addition, an add(19)(p13) was shown to result from a nonreciprocal translocation der(19)t(19;20)(p13.3;p?q?). The karyotype of MCC26 also showed a del(2)(p21) in all metaphases and an add(2)(p24) present in half of the analyzed mitoses. M-FISH showed that the add(2)(p24) resulted from a der(2)t(2;9)(p24;q12) and confirmed the presence of a normal chromosome 2 and a chromosome 2 with a deletion in the short arm. The CGH profile is in agreement with the presence of an interstitial 2p deletion partially masked by the der(2)t(2;9)(p24;q12) in half of the cells (Fig. 1b). Combination of CGH and M-FISH showed that two almost identical marker chromosomes were derived from the short arm of chromosome 5 (Fig. 1c). In addition, M-FISH also revealed cryptic translocation with partner chromosomes 18 and 22, respectively for these two derivative chromosomes 5. An add(5)(pter) was identified as a der(5)t(5;11)(p15;q14.2) (Fig. 1c). A third marker chromosome was characterized by M-FISH and CGH as a del(12)(q12) as CGH showed overrepresentation for the 12p region (data not shown).

The karyotype of MKL-1 subclone 1 was initially published by Rosen *et al.*[4]. Two i(5)(p10) chromosomes and one der(7)t(7;8)(q36;q22) were described. The presence of two

isochromosomes 5p was confirmed by M-FISH whereas the derivative 7 was revised as a der(7)t(7;11)(q36;q14) (data not shown).

In MKL-1 subclone 2, M-FISH confirmed the presence of the der(1)t(1;17)(p36;q21).[4] Deletion of the most distal part of 1p was suggestive by CGH in addition to 17q gain (Fig. 1d). FISH with region specific probes showed indeed that the 1;17 translocation resulted in a small distal deletion including markers D1Z2 (1p36.33), *TP73* (1p36.33) and *DFFB* (1p36.3) whereas the *NPPA* gene (1p36.31-p36.23) was retained. In addition, M-FISH identified a cryptic translocation der(10)t(10;12)(q23;p11.21) which was confirmed by CGH (data not shown).

In cell line MKL-2, complex translocations involving chromosomes Y, 1, 3, 11, 13, 14, 16, 17, 21 and 22 were identified by M-FISH with chromosome 3 and 13 material being translocated onto at least 3 different partner chromosomes (Table 1 and Fig. 1e). In addition, some of the unbalanced translocations seemed to involve a "jumping" or "segmental jumping" translocation.[59,60] The donor segment 3q13.3~21→qter was translocated to more then one recipient chromosome: der(1) and der(17) and a part of this segment (3q23-q26) seemed additionally translocated to the der(11) resulting in gain of 3q23-q26 material in MKL-2. FISH with 1p-specific probes showed translocation of markers D1Z2, *TP73*, *NPPA*, A12M2, *ID3* and *EXTL1* to the derivative chromosome 3. In addition, an extra copy of the *Connexin27* (1p35.1) and *MYCL1* (1p34.3) were present on the derivative chromosome 17 (data not shown). FISH with the LSI SRY/CEPX probe showed that the most distal part of the short arm of chromosome Y was lost in this derivative chromosome 17 (data not shown). In addition two marker chromosomes were identified as an i(4)(p10) and a der(20)(20pter→20q13::4q13→4qter), respectively. An unbalanced rearrangement der(6)t(6;8)(q16;q22) was identified by M-FISH.

The karyotype of cell line UISO, described by Ronan *et al*.[12], was previously revised by us and revealed insertion of material of unidentified origin to 1p36.2[20] and 6q, respectively. Interestingly, the 1p36.2 breakpoint mapped within a cluster of repetitive tRNA and snRNA genes[20] and coincided with the constitutional 1p36 breakpoint previously reported in a patient with neuroblastoma.[61,62] In addition, a ring chromosome and a der(8) were also present in UISO. M-FISH combined with CGH identified the der(8) as a dic(8;8)(8qter→8p21.2::8p21.2→8qter) but could not reveal the nature of the insertions and the ring chromosome. Apparently the inserted sequences and the ring chromosome contained hybridization signals which were positive for each fluorochrome. Since a combination of all five fluorochromes is not present in any of the used chromosome paints, we assumed that this pattern could be explained by the presence of repetitive DNA sequences. To test this hypothesis FISH was performed under low stringent conditions with probes for the heterochromatic regions of chromosome 1, 9 and 16 and the alpha satellite DNA probe p82H. Using this probe mixture, hybridization signals were detected on the derivative chromosomes 1 and 6 and the ring chromosome (Fig. 1f) whereas hybridization with D1S80 for the short arms of the acrocentric chromosomes was negative (data not shown). The FISH hybridization patterns point to the presence of at least two different kinds of repetitive DNA material i.e. satellite III repeat units and alpha satellite repetitive sequences. The significance of these findings is presently unclear and warrants further investigation.

Karyotyping of tumor UHG-RM revealed structural abnormalities of chromosomes 3, 9, 15 and 22 (Fig. 1g). M-FISH analysis showed that these chromosomes were involved in one single apparently balanced but complex translocation with at least eight breaks and cryptic involvement of chromosome 17 (Table 1, Fig. 1h). FISH with gene specific probes for *SHOX2*, *ABL* and *BCR* was applied to define the 3q25-q26.1, 9q34 and 22q11

breakpoints in these translocations. Deletion of chromosome 10 was the only numerical change observed in the tumor. Previous mutation analysis of the *PTEN* (10q23) gene in this case was negative.[28]

Discussion

The number of cytogenetic reports (for overview see Table 2) and molecular investigations on Merkel cell carcinomas (MCC) are limited. Recurrent losses have been reported for chromosomal regions 1p, 3p, 9p, 10q and 13q and gains were noticed for 1q, 3q, 5p, 6, 8q, 19 and X[18,26,63,27,25,64,23,28,29] but further genetic studies are necessary to obtain a more detailed picture of the genetic changes that lead to MCC development. To this purpose, we combined karyotyping, CGH, M-FISH and FISH with region specific probes to analyze 6 MCC cell lines and one metastatic MCC tumor. This approach yielded a comprehensive and improved description of chromosomal changes which were either not detected or only partially identified or classified as markers by G-banding alone. The chromosomes most frequently involved in translocation events were in order of frequency 3, 1, 5, 7, 13, 14, 8, 9, 17 and 22. Eight breakpoints in the chromosome three long arm were detected in three of the seven cases. Four breakpoints were observed in two MCCs, each at 3q23 and 3q25-q26.1. Three additional breakpoints were located more proximal at 3q11.2, 3q13.1 and 3q13.3 whereas one was located more distal at 3q29.

Gain of 3q material has been recognized as a recurrent imbalance in MCC[25] and was here observed in one MCC. In this case, the region of gain was restricted to 3q23-q26 material, thus defining a relatively small critical region for 3q gain. We propose that this region harbors a gene that due to dosage effect of a wild-type or mutated allele may contribute to MCC oncogenesis. A possible candidate gene located in this region is the *ATR* (ataxia-telangiectasia and Rad3-related) gene. Gain of this gene due to isochromosome 3 formation led to loss of differentiation and cell cycle abnormalities in rhabdomyosarcomas.[65] Expression and/or mutation studies of the *ATR* gene should reveal if this gene is also implicated in MCC and other tumor types with 3q gain.

Previous cytogenetic studies of MCC showed that structural rearrangements of chromosome 1, often leading to loss of 1p material, occurred in more than half of the reported cases. The observation of two MCC tumors with chromosome 1p rearrangements as the sole structural chromosomal abnormality[14,20] provided further evidence for a role of tumor suppressor genes on 1p36 in MCC pathogenesis. Here, molecular cytogenetic analysis of an unbalanced 1;17 rearrangement in MKL-1 (subclone 2) maps the 1p breakpoint between *DFFB* and *NPPA* within band 1p36.3. This deletion breakpoint coincides with a previously described deletion breakpoint by Van Gele *et al*.[20]. The *TP73* gene was considered in many neoplasms as a strong candidate gene for this deleted region, but mutation studies provided no evidence to support this view, indicating that the *TP73* gene is probably not the primary target of deletions in this region or is inactivated by alternative mechanisms.[66,67]

A second interestingly observation was made in cell line MKL-2 which showed a complex 1p rearrangement. Distal 1p material with a breakpoint between the *EXTL1* (1p36.1) and the *Connexin27* gene (1p35.1) was translocated to 3q13.3~21. In addition, a small interstitial chromosome 1 segment containing *Connexin27* and *MYCL1* (1p35.1→1p34.3) was translocated to a der(17) together with chromosome 3 and chromosome Y material. This type of complex rearrangements may explain some of the unusual LOH patterns observed by Leonard *et al*.[23].

M-FISH analysis revealed two isochromosomes of the short arm of chromosome 5 and two derivative 5 chromosomes containing mainly 5p material in MKL-1 (subclone 1) and MCC26, respectively. The occurrence of i(5p) and other isochromosomes have been reported previously in MCC[3,5,8,11,15,25]and could correlate to a low-level copy number gain of genes, which interfere with growth advantage. A possible candidate gene located at 5p15 is *hTERT* (human telomerase reverse transcriptase).[68] Regulation and/or activation of the *hTERT* gene leads to telomerase activity following cellular immortalization, which is an important step in the development of cancer.[69] One of the possible mechanisms contributing to the dysregulation of telomerase activity observed in human tumors could be an increased expression of *hTERT* through gain of 5p material. This was demonstrated in a study by Zhang *et al*.[70] in which a number of human tumor cell lines and primary tumors acquired extra *hTERT* gene copies resulting in an enhanced gene expression and telomerase activity, possibly due to gain of 5p material. Recently, telomerase activity was also observed in Merkel cell carcinoma suggesting a correlation between telomerase activity and its malignant development.[71] In this respect it is of interest that telomerase suppressor genes may be located at two different regions on chromosome 3p (3p21.3-p22 and 3p12-p21.1).[72] Deletion of such genes located in these regions could cause derepression of *hTERT* and lead to telomerase activity in proliferating tumor cells. A previously performed LOH and CGH study showed frequently loss of 3p in MCC and identified a critical region of loss at 3p13-p14.[26,25]Loss of a possible telomerase suppressor gene located in this region could also contribute to telomerase activity in a subset of MCCs.

A previous study demonstrated that *PTEN* was involved at low frequency in MCC, indicating that probably other tumor suppressor genes located at or distal to 10q23 could be involved.[28] In this study, two additional cases with whole chromosome 10 loss were observed and loss of 10q23→qter was seen in one MCC. A 10q23 deletion breakpoint was also observed in a metastatic MCC tumor described by Schlegelberger *et al*.[13]. Further studies of *PTEN* and other candidate genes in these cases should reveal their involvement in MCC development.

In conclusion, this is the first report describing M-FISH analysis of MCC in combination with CGH, conventional FISH and banding analysis. Using this approach, the genetic changes in 6 MCC cell lines and one metastatic MCC tumor were characterized. We identified novel translocations widely distributed throughout the genome, including complex chromosome 3q and 1p rearrangements. Our accurate cytogenetic profiling of the chromosomal changes contributes greatly to the limited cytogenetic data of MCC and opens new perspectives in the search for candidate tumor suppressor genes or oncogenes in these chromosomal regions. Understanding the molecular basis of these chromosomal events would finally lead to a better insight in the mechanisms affecting and regulating this malignant disease.

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Table 1. Karyotype description based upon banding analysis only and revised karyotypes based upon M-FISH analysis combined with banding, FISH with region specific probes and CGH data.

Cases	karyotype	M-FISH/CGH/FISH revised karyotype
MCC13	46,XX,del(7)(q22),der(8)t(8;?)(q?;?),add(9)(q34),del(12)(q12q14)[6], -13,add(14)(q32),+mar[cp12]	46,XX,t(7;8;14)(7pter→7q11.23::14q31.1→qter;8pter→8q23::7q11.23→7q31.1::8q23→ 8qter;14pter→14q31.1::7q31.1→7qter),der(9)(9pter→9q34::13q22.2→13qter),der(13) (13pter→13q12.3::16q23q24→16qter),del(12)(q12q14)
MCC26	58~61,XX,+del(X)(q?24),+add(1)(q23),del(2)(p21),+add(2)(p24)[5], add(3)(q26),der(4)t(1;4)(q12;p16),add(5)(pter),+6,+8,del(11)(q14.2),+12[4],+13,add(14)(p11),+15,+18,add(19)(p13),+20,+20,der(21)t(14;21)(q12;p1 1),+22,+3mar[cp10]	60,XX,+der(X)(Xpter→Xq?::16p?q?→16p?q?),+der(1)(1pter→1q23::3q23→3qter),del(2)(pt er→p13::p16→qter),+der(2)(:2p24;9qter→9q12),der(3)(3pter→3q23::7q21→7qter),der(4)(1 qter→1q12::4p16→4qter),der(5)(5p15;11qter→11q14.2),+der(5)(5pter→5q12;18qter→18q?),+der(5)(5pter→5q12;22p?q?),+6,+8,del(11)(q14.2),+del(12)(q12),+12,der(14)(8p?q?→8p? q?::Xq?→Xq?::14p11.1→14qter),+15,+18,der(19)t(19;20)(p13.3;p?q?),+20,+20,+der(21) (21qter→21p11.2::14q12→14qter),+22
MKL-1 (subclone 1)	48,XY,i(5)(p10)x2,add(7)(q36)	48,XY,i(5)(p10)x2,der(7)t(7;11)(q36,q14)
MKL-1 (subclone 2)	45~46,XY,der(1)t(1;17)(p36;q21),-8,-14[5][cp10]	46,XY,der(1)t(1;17)(p36.3;q21.3),-8,der(10)t(10;12)(q23;p11.21)
MKL-2	41~42,X,-Y,add(1)(p36),-3,del(3)(q13),-4,del(6)(q21q23),-10,-11, add(11)(q23),-13,add(14)(p11.2),add(16)(q24),-17,-20,-21,-22, +3~4mar	41,X,der(1)(3qter→3q13.3~21::1p35.1→1qter),der(3)(3pter→3q13.3~21::1p36.1→1pter), -3,i(4)(p10),der(6)(6pter→q16;8q22→8qter),-10,der(11)(11pter→11q24::3q23→3q25-q26.1 ::13q?→13q?::3q29→3qter),-13,der(14)(21qter→21q21::3p?q?→3p?q?::13q?→13q?:: 14p11.2→14qter),der(16)(16pter→16q24::13q?→13qter),der(17)(Yqter→Yp11.2::17q11.1 →17qter::1p35.1→1p34.3::3q13.3~21→3qter),der(20)(20pter→20q13::4q13→4qter),-21 der(22)(22pter→22p11.2::3q11.2::22p11.2→22qter)
UISO	46~47,XX,ins(1;?)(p36.2; ?),ins(6;?)(q15;?),der(8)t(8;8)(p12;q11.2), +r(?)	45~46,XX,ins(1;?)(1pter→1p36.2::1q12::?::1p36.2→1qter),ins(6;1;?)(6pter→6q15::1q12::?:: 6q15→6qter),dic(8;8)(8qter→8p21.2::8p21.2→8qter),der(1)r(1;?)(q12::?)
UHG-RM	45,XX,del(3)(q13.1),add(9)(q34),-10,del(15)(q21),add(22)(q12)	45,XX,t(3;9;15;17;22)(3pter→3q13.1::22q12→22qter;9pter→9q13::3q13.1→3q25-q26.1:: 9q13→9q34::15q21.1→15qter;15pter→15q21.1::3q25-q26.1→3qter;17pter→17q24::9q34 →9qter;22pter→22q12::17q24→17qter),-10

Table 2. Literature overview of chromosomal abnormalities in Merkel cell carcinoma

Reference	Case	Tumor Type	Modal number	Structural and numerical abnormalities
Kusyk et al., 1986	1	LNM[a]	54-64	+X,del(1)(q?23q?24)x2,i(13q),der(15)t(15;21)(q13;q25),+6,+7,+9,+10,+11,+18,+19,+20,+mar
Hopfner et al., 1987	2	P[b]	47	i(8q),+6,-10,+12
Rosen et al., 1987	3	CL[c]	47-48	der(1)t(1;17)(p36;q21),i(5p)x2,der(7)t(7;8)(q26;q22)
Sozzi et al., 1988	4	P	45	inv(1)(p33q11),+del(1)(p22p33),+1
	5	LNM	48	der(1)t(1;?)(p36;?),+1,+3,+6
	6	LNM	49	i(7p),del(11)(p13p14),der(15)t(15;?)(p12;?),+6,+11,+12
	7	LNM	44	del(1)(p22p32)(q41q44),del(3)(q13q29),+1
	8	P, LNM	54	del(1)(q31q44),t(1;3)(q31;q26),+1,+9,+10,+11,+12,+14,+18,+19,+20,+21,+4mar
Sandbrick et al., 1988	9	LNM	48	del(7)(q31.2),+6,+11
Shabtai et al., 1989	10	LNM	46	del(2)(p11),del(22q)(q12),-5,-14,-17,+20,-22
Koduru et al., 1989	11	LNM	46	der(1)t(1;13)(q21;p13),del(5)(q31)
	12	LNM	46	i(1q),der(2)t(2;?)(q37;?),i(6p),der(10)t(10;?)(p15;?),der(17)t(17;?)(p13;?),-4,+mar
	13	P, LNM	47	der(1)t(1;6)(q22;p25),der(21)t(21;?)(p13;?),+12,-20
English et al., 1990	14	LNM	47	+7 or add(7)(q?)
Smadja et al., 1991	15	BMM[d]	46	dup(1)(q11q44),t(2;3)(p13;q?26q?27),der(15)t(15;?)(p12;?)
Ronan et al., 1993	16	CL	46	t(1;17)(p36;q21),t(1;6)(p22;q26),r(8),+2,-8,-17
Leonard et al., 1993	17	LNM	43	der(1)t(1;5)(p36;p13),i(11p)(p10),-1,-11,-13,-22,+mar
	18	REC[e]	49	+X,-Y,add(7)(p22),i(9q),i(11p),-7,-9,-11,+14
	19	P	44	del(1)(p22),del(1)(q21),der(1)(1;?)(q21;?),-1,-3,-11,-13,-13,+18
	20	LNM	55	+1,-8,+7mar
	21	REC	47-49	+1,-5,-8,-13,-13,+5mar
	22	REC	43-44	-9,-13,-22
Schlegelberger et al., 1994	23	LNM	46-48	der(X)t(X;10)(p22;q23),t(1;15)(q12;p13),t(1;16)(q21;p13),t(1;14)(p12;q32),add(10)(p11),+18,+19
Gibas et al., 1994	24	P	46	del(1)(p36.1)
Tope et al., 1994	25	P	46-48	-Y,+5,+7,+18
Moll et al., 1994	26	CL	45-50	i(5p)x2,add(1)(p?),t(1;12)(p13;q13),del(1)(p?),del(2)(q33),add(6q),add(12q)
	27	CL	49	del(11)(q21),t(5;17)(p15;p13),t(1;7)(q32;p15p),+6,+20,+Y
Perlman et al., 1995	28	PM[f]	46	der(1)t(1;3;22)(p34;q28-->q11;q12),der(3)t(1;3)(p35;q11),der(22)t(3;22)(q29;q11)
Vazquez-Mazariego et al., 1996	29	LNM	46	der(6)t(1;6)(q23;q27),der(9)t(1;9)(q11;p24)
Larsimont et al., 1996	30	P	47	+6
Van Gele et al., 1998	31	P	46	der(1)t(1;1)(p36.31;q12)
	16	CL	47	ins(1;?)(p36.2;?),ins(6;?)(q15;?),dic(8;8)(8qter-->8p21.2::8p21.2-->8qter),+r?

[a]Lymph node metastasis; [b]Primary tumor; [c]Cell line; [d]Bone marrow metastasis;[e]Recurrence at site; [f]Pulmonary metastasis

LEGENDS TO THE FIGURES

Fig. 1 Selection of M-FISH findings in relation to karyotypic, FISH and/or CGH results.

a. Partial karyotypes and M-FISH images of selected chromosomal rearrangements for MCC13 illustrating the three-way translocation t(7;8;14)(7pter→7q11.23::14q31.1→ qter;8pter→8q23::7q11.23→7q31.1::8q23→8qter;14pter→14q31.1::7q31.1→7qter) (top) and the der(9)(9pter→9q34::13q22.2→13qter) and der(13)(13pter→13q12.3:: 16q23q24→16qter) (bottom).
b. Partial karyotypes and M-FISH images of unbalanced chromosome 2 rearrangements (top) and two normal chromosomes 9 (bottom) in MCC26 showing a del(2)(pter→p13::p16→qter) and a der(2)(:2p24;9qter→9q12). The CGH profile of chromosome 2 shows a consistent but statistically not significant deviation of the ratio profile at 2p13→2p16 and a statistical loss of the very distal (2p24) part of chromosome 2.
c. CGH (right) combined with banding (left bottom) and M-FISH (middle top) led to the identification of two der(5) chromosomes derived from the short arm of chromosome 5 and involved in cryptic translocations namely a der(5)(5pter→5q12;18qter→18q?) and a der(5)(5pter→5q12;22p?q?) (bottom) together with a der(5)(5p15;11qter→11q14.2) and del(11)(q14.2) (top) in MCC26. Chromosomes 5, 18 and 22 are displayed in red, green and blue colors respectively, chromosome 11 in grey (bottom).
d. Unbalanced 1;17 translocation (left), M-FISH (middle) and CGH profiles (right) showing loss of distal 1p and overrepresentation of 17q material in MKL-1 (subclone2).
e. M-FISH image of a complete karyotype of cell line MKL-2.
f. Partial karyotypes and inverted black and white FISH images of the normal chromosome 1, the derivative 1, the normal chromosome 6, the derivative 6 and the ring chromosome in cell line UISO. Probes used for the 1q12 region (D1Z1) are shown in red and for p82H, pHUR195 and pHUR98 in green.
g. Complete G-banded karyotype for MCC tumor UHG-RM.
h. M-FISH image of a complete karyotype of MCC tumor UHG-RM.

Fig. 2 Overview of chromosomal breakpoints and imbalances detected by karyotyping, FISH, CGH, and M-FISH.

CGH findings were available for MCC13 (1), MCC26 (2), UISO (6), and UHG-RM (7). CGH was performed in this study for MKL-1 (subclone 2) (4). CGH data were not available for MKL-1 (subclone 1) (3) and MKL-2 (5).
The vertical lines on the left side of the chromosome ideogram indicate underrepresentation, those on the right side overrepresentation of the corresponding chromosomal regions. Dashed lines were used to indicate borderline CGH profile deviations. The position of chromosomal breakpoints as determined by a combination of M-FISH with banding analysis or CGH is indicated by an open circle. A filled circle means that the position of the breakpoint could not be defined exactly. An empty arrowhead indicates the location of an insertion breakpoint. Open squares are used to indicate the position of the chromosomal breakpoints as determined by a combination of M-FISH with banding analysis for the cell lines for which no CGH data were available. Filled squares are used if the position of the breakpoint could not be defined exactly.

Figure 1.

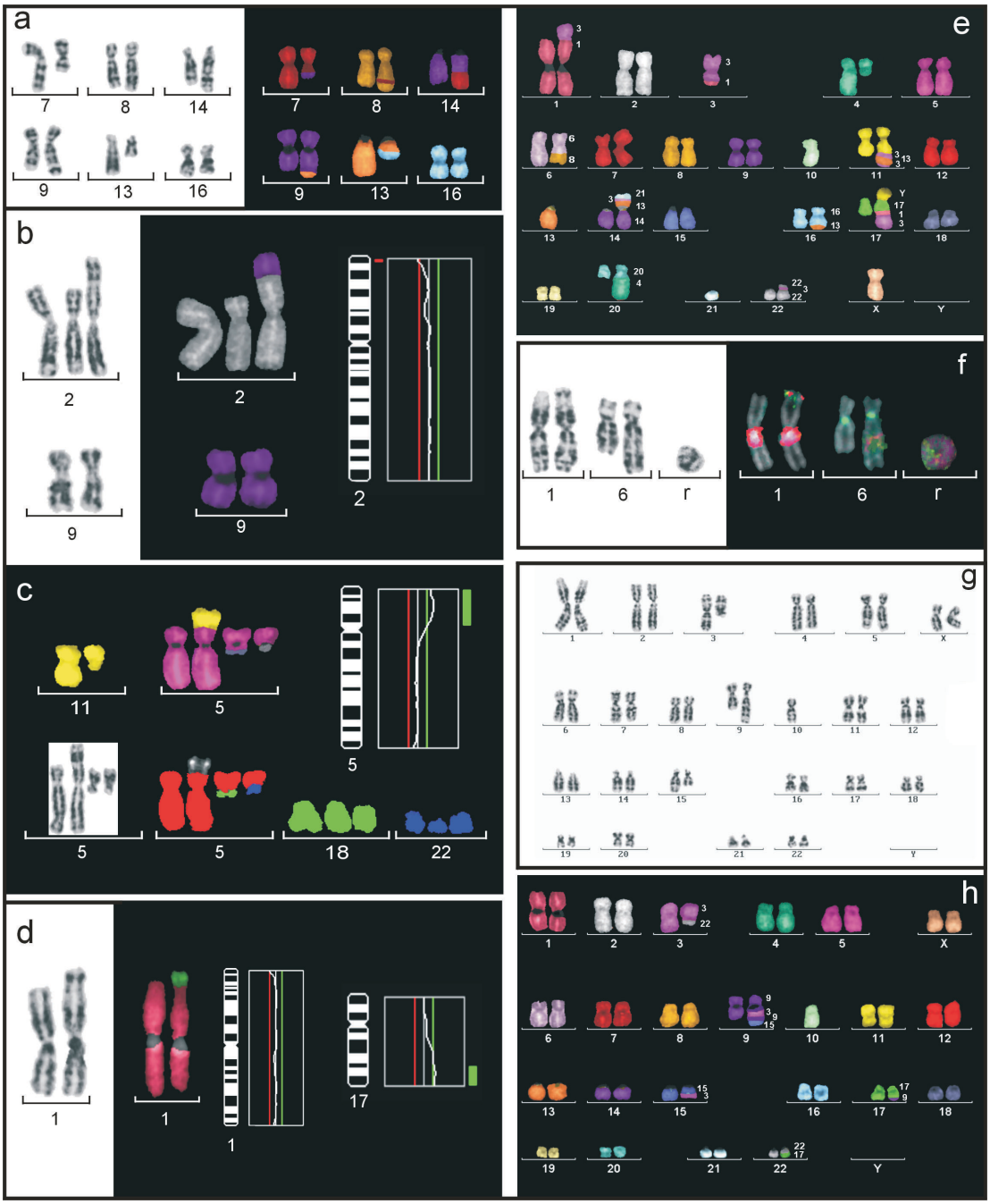
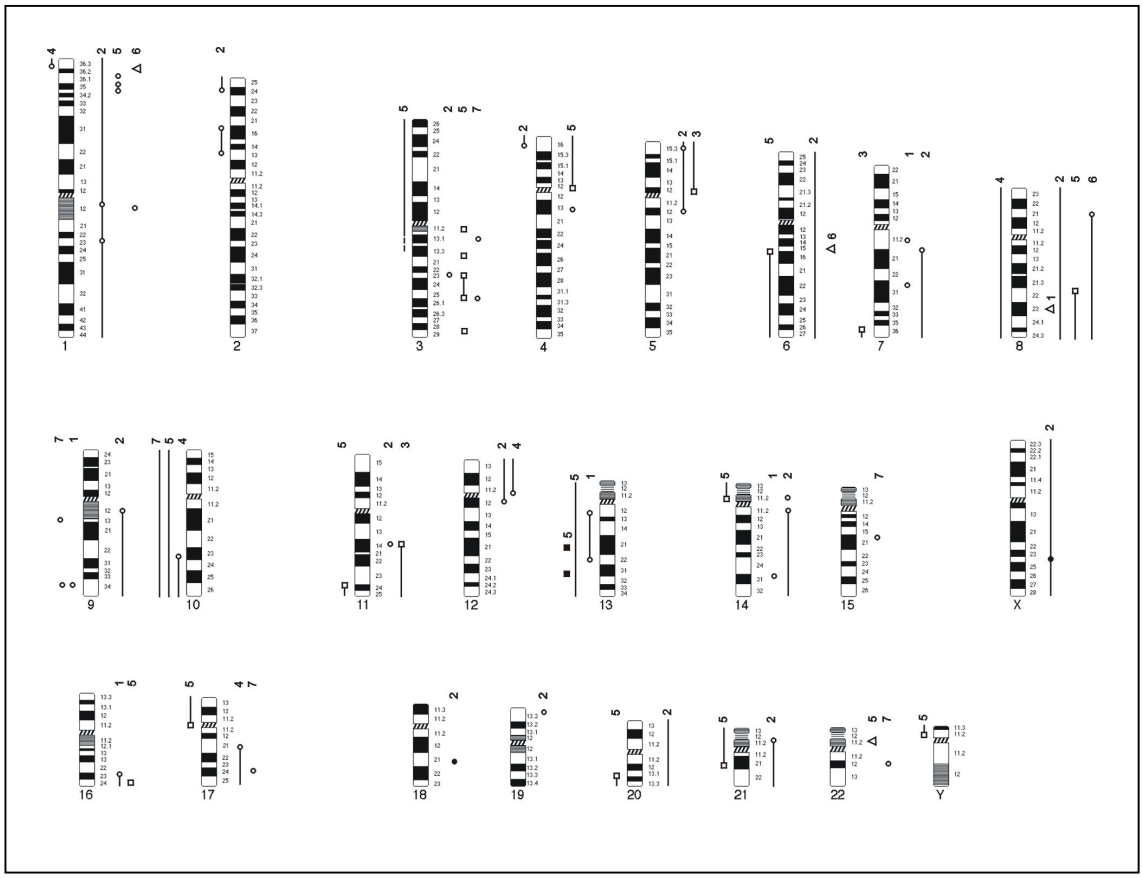


Figure 2.



PART 3

Candidate Gene Analysis

3.1 Mutation analysis of candidate tumor suppressor genes

3.1.1 Mutation analysis of *P73* and *TP53* in Merkel cell carcinoma

Van Gele M., Kaghad M., Leonard J. H., Van Roy N., Naeyaert J. M., Geerts M. L., Van Belle S., Cocquyt V., Bridge J., Sciot R., De Wolf-Peeters C., De Paepe A., Caput D., Speleman F. *Br J Cancer, 82: 823-826, (2000).*

3.1.2 Frequent allelic loss at 10q23 but low incidence of *PTEN* mutations in Merkel cell carcinoma

Van Gele M., Leonard J. H., Van Roy N., Cook A. L., De Paepe A., Speleman F. *Int J Cancer, 92: 409-413, (2001).*

3.1.1 Mutation analysis of *P73* and *TP53* in Merkel cell carcinoma

TP73 (alias *P73*) was originally described as a monoallelic expressed gene and assigned to 1p36.33 (Kaghad *et al.*, 1997). This region is frequently deleted in many tumor types, including neuroblastoma, and is believed to harbour an imprinted tumor suppressor gene (Caron *et al.*, 1995). Interestingly, *TP73* showed significant sequence similarity to the transactivation (29%), DNA-binding (63%) and oligomerization domain (38%) of the *TP53* gene (Kaghad *et al.*, 1997). A study by Jost *et al.* (1997) showed that overexpression of *TP73* in SAOS-2 cells ($TP53^{-/-}$) could activate the transcription of *TP53*-responsive genes like $p21^{waf1/cip1}$ and inhibited cell growth in the same way as *TP53* by inducing apoptosis. However, *TP73* was not directly induced in response to certain DNA-damaging stimuli. Instead, the tyrosine kinase *c-abl* interacted and phosphorylated *TP73* on tyrosine residue 99 and induced *TP73*-mediated transactivation and apoptosis (Figure 3-1) (Agami *et al.*, 1999; Yuan *et al.*, 1999).

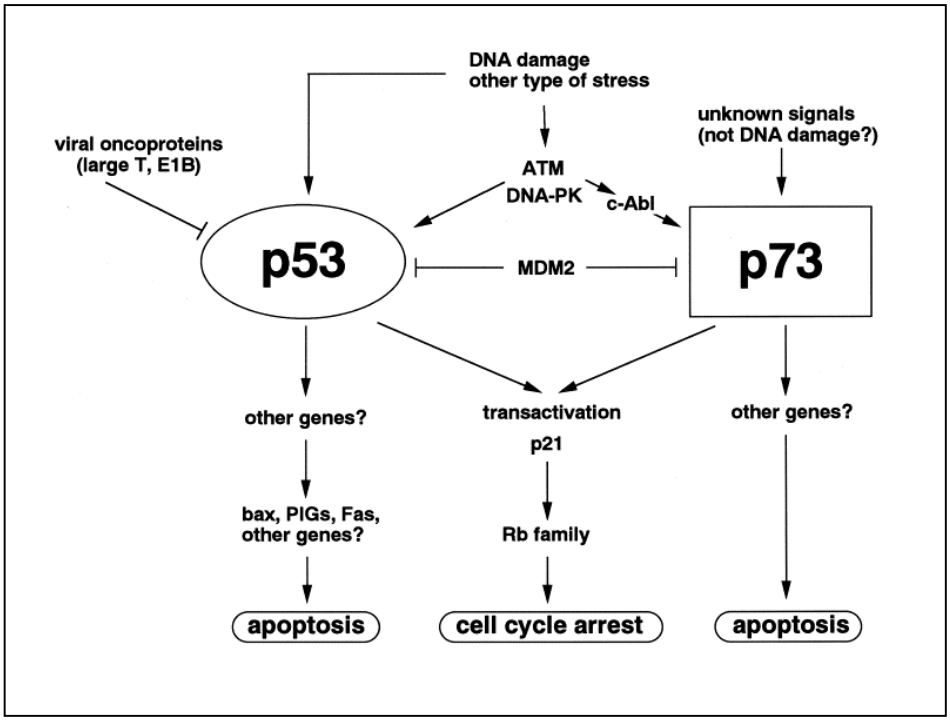


Figure 3-1: Diagram showing pathways of *TP53* and *TP73* to induce cell cycle arrest and apoptosis in response to various stimuli.

(Reproduced from Ichimiya et al., Path Int., 50:589-583, 2000)

In view of the frequent occurrence of distal 1p deletions in MCC, we screened the *TP73* gene for inactivating mutations in five MCC cell lines and ten MCC tumors. Given the possible complementary functions of *TP73* and *TP53*, the mutation status of *TP53* was also examined. A *TP73* missense mutation, which could possibly reduce the transcriptional transactivation function of the amino-terminal region of *TP73*, was detected in one MCC tumor. These results show that *TP73*, analogous to other cancers, is infrequently mutated in MCC. In addition, four *TP53* mutations located at mutational hotspot regions were identified in two MCC cell lines and one *TP53* non-sense mutation was found in a MCC tumor. This report showed for the fist time inactivating *TP53* mutations in MCC. The finding of typical UVB induced mutations in one MCC cell line provided further evidence for sun-exposure in the etiology of this skin tumor.

Presently, their is evidence however that several TP73 isoforms exist of which some may exert a dominant negative effect on TP53 or TP73 activity (Yang *et al.*, 2002). This provides circumstantial evidence that *TP73* is involved in tumorigenesis. Future investigations should reveal if some of these isoforms play a role in MCC oncogenesis (see General Discussion).

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Mutation analysis of *P73* and *TP53* in Merkel cell carcinoma

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Summary The *p73* gene has been mapped to 1p36.33, a region which is frequently deleted in a wide variety of neoplasms including tumours of neuroectodermal origin. The p73 protein shows structural and functional homology to p53. For these reasons, *p73* was considered as a positional and functional candidate tumour suppressor gene. Thus far, mutation analysis has provided no evidence for involvement of *p73* in oligodendrogliomas, lung carcinoma, oesophageal carcinoma, prostatic carcinoma and hepatocellular carcinoma. In neuroblastoma, two mutations have been observed in a series of 140 tumours. In view of the occurrence of 1p deletions in Merkel cell carcinoma (MCC) and the location of *p73* we decided to search for mutations in the *p73* gene in five MCC cell lines and ten MCC tumours to test potential tumour suppressor function for this gene in MCC. In view of the possible complementary functions of *p73* and *TP53* we also examined the status of the *TP53* gene. Sequence analysis of the entire coding region of the *p73* gene revealed previously reported polymorphisms in four MCCs. In one MCC tumour, a mis-sense mutation located in the NH_2-terminal transactivation region of the *p73* gene was found. These results show that *p73*, analogous to neuroblastoma, is infrequently mutated in MCC. This is also the first report in which the role of *TP53* in MCC has been investigated by sequencing the entire coding region of *TP53*. *TP53* mis-sense mutations and one non-sense mutation were detected in three of 15 examined MCCs, suggesting that *TP53* mutations may play a role in the pathogenesis or progression of a subset of MCCs. Moreover, typical UVB induced C to T mutations were found in one MCC cell line thus providing further evidence for sun-exposure in the aetiology of this rare skin cancer.

Keywords: Merkel cell carcinoma; 1p36; *p73*; *TP53*; mutation

Merkel cell carcinoma (MCC) is a rare aggressive neuroendocrine skin tumour, mostly affecting elderly individuals. The majority of primary MCCs occur on sun-exposed areas of the skin, implicating UV exposure in its aetiology. The tumour is thought to originate from Merkel cells, which have properties of both epithelial and neuroendocrine cells. They are located in the basal epidermis and are likely to play a role in signal transduction as receptors for mechanical stimuli in all animals (Ratner et al, 1993).

Until now, little was known about the genetic basis of MCC. Only 13 cytogenetic studies have been reported in the literature (Mitelman et al, 1998). No consistent translocations have been observed but structural abnormalities involving the short arm of chromosome 1 have been noted in 40% of all examined cases. Chromosome region 1p36, which is frequently deleted in a variety of neoplasms including neuroendocrine tumours such as neuroblastoma, is often affected in MCC tumours with 1p rearrangements (Van Gele et al, 1998). Several candidate tumour suppressor genes have been mapped to 1p36 but as yet no direct involvement of these genes in tumours with 1p36 loss could be found (Schwab et al, 1996).

The *p73* gene, located on 1p36.33, has also been considered as a candidate tumour suppressor gene. *p73* shows significant sequence similarity to the transactivation, DNA-binding and oligomerization domain of *TP53* (Kaghad et al, 1997). Jost et al (1997) showed that overexpression of *p73* can activate the transcription of *TP53*-responsive genes like $p21^{waf1/cip1}$ and inhibit cell growth in the same way as *TP53* by inducing apoptosis. Mutation analyses for the *p73* gene have been performed in lung carcinoma, oligodendrogliomas, prostatic carcinoma, neuroblastomas, colorectal carcinoma, oesophageal carcinoma and hepatocellular carcinoma (Kovalev et al, 1998; Mai et al, 1998*a*, 1998*b*; Nimura et al, 1998; Nomoto et al, 1998; Sunahara et al, 1998; Takahashi et al, 1998; Ichimiya et al, 1999; Mihara et al, 1999). Except for two mutations in primary neuroblastomas resulting in amino acid substitutions in the C-terminal region of p73 (Ichimiya et al, 1999), no other mutations have been reported.

We decided to perform mutation analysis of the *p73* gene in order to investigate the possible involvement of *p73* in MCC oncogenesis. Five MCC cell lines and ten MCC tumours with known 1p status were examined for mutations in the *p73* gene by direct sequence analysis. In view of the possible complementary functions of *p73* and *TP53*, the status of *TP53* was also investigated.

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MATERIALS AND METHODS

Samples

Tumour samples were collected at the University Hospitals of Ghent and Leuven, Belgium and the University of Nebraska

Medical Center, Ohama, NE, USA. One part of the sample was snap-frozen in liquid nitrogen and another part was stored at –80°C until analysis. MCC tumour cell line UISO was kindly provided by Dr SG Ronan and Prof Dr TK Das Gupta (Illinois, USA). Cell line MKL-1 was given by Prof Dr ST Rosen (Illinois, USA). Cell lines MCC13, MCC14/2 and MCC26 were a gift from Dr JH Leonard (Queensland, Australia). All cell lines were grown in RPMI-1640 medium (Gibco-BRL, 52400-025). DNA of the tumour samples and cell lines was prepared by standard proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation protocols. Constitutional DNA of the patients was isolated by using the Qiagen blood and cell culture Midi Kit (Westburg, 13343). RNA extraction from the tumour samples and cell lines was done with RNeasy Midi Kits (Westburg, 75144).

Fluorescence in situ hybridization

In order to determine the copy number of chromosome 1 and presence or absence of 1p deletions, fluorescence in situ hybridization (FISH) was performed on metaphases or interphase nuclei according to Van Roy et al (1994). DNA probes used were pUC1.77 (*D1Z1*) for the heterochromatic region of chromosome 1 (1q12) and p1–79 (*D1Z2*) for the subtelomeric region of chromosome 1 (1p36.33).

Reverse transcriptase polymerase chain reaction and sequencing analysis

cDNA synthesis was performed using 5 µg of total RNA incubated in a 20 µl volume reaction containing 50 mM Tris–HCl (pH 8.3), 10 mM dithiothreitol (DTT), 10 mM potassium chloride (KCl), 0.5 mM dNTP, 20 U RNAsin (Promega), 150 U Super-script II Reverse Transcriptase (Gibco-BRL) and 50 ng μl^{-1} oligo-d(T) primer for 1 h at 37°C. The primer pairs sense: 5′-AGGGGACGCAGCGAAACC; antisense: 5′-GGCAGCTTG-GGTCTCTGG; and sense: 5′-GGTGACACGCTTCCCTG; antisense: 5′-GGTGGGAGGCTGTCAGTG were used to amplify the entire coding region of *p73* and *TP53* respectively by reverse transcription polymerase chain reaction (RT-PCR). Long range PCR was performed using the Expand™ Long Template PCR system (Boehringer Mannheim). PCR reactions were performed in a 50 µl reaction volume containing 2 µl of reverse transcriptase products, 500 µM dNTP, 300 nM primers, 10% dimethylsulphoxide, 5 µl of buffer 1 and 3.5 units of *Taq* and Pwo enzyme mix. The PCR amplification consisted of 35 cycles of 95°C for 0.5 min, 58°C for 1 min, 68°C for 2.5 min after starting with a denaturing step at 95°C for 1 min and ending at 68°C for 10 min. The PCR products were purified by spin dialysis sequentially on S400 and P10 resins. Subsequently, the PCR products were directly completely sequenced on an ABI PRISM™ 377 sequencer (Perkin-Elmer) using specific nested sequencing primers and the Big Dye Terminator Sequencing Kit. Cases 6, 7, 8, 9 and 10 (see Table 1) were sequenced on an ALF™ Express automated DNA sequencer (Pharmacia) using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham, RPN 2438). To confirm the *p73* nucleotide change found in case 4 the following primer pair sense: 5′-CCAGTTCAATCTGCTGA-GCAG-3′ and antisense: 5′-CCACTTTGAGGTCACTTTCC-3′, located in exon 4, were used to amplify genomic tumour DNA and constitutional DNA of the patient. PCR conditions were the same as those described above. The PCR products were sequenced using the same primers as those to amplify genomic DNA.

Table 1 Summary of mutation analysis of *p73* and *TP53* in Merkel cell carcinoma

Cell lines	1pΔ[a]	Expressed allele(s)[b]	p73 Transcript sequence	AA change	TP53 Transcript sequence	AA change
UISO	No[c]	G/C	wt	–	wt	–
MKL-1	No	G/C	wt	–	wt	–
MCC13	No	G/C;A/T	wt	–	mut 857C>T;858C>T	S241F
					mut 967C>T	P278S
MCC14/2	No	G/C	wt	–	mut950T>A	V272E
MCC26	No	G/C	wt	–	N/A[d]	
MCC samples						
Case 1	No	G/C;A/T	1118C>T;1157T>C[e]	No	wt	–
			1781G>A;1940G>A	No		
Case 2	Yes	A/T	1118C>T;1157T>C	No	wt	–
			1781G>A;1940G>A	No		
Case 3	No	G/C;A/T	1118C>T;1157T>C	No	wt	–
			1781G>A;1940G>A			
Case 4	No	G/C;A/T	mut 439C>T	S110L	mut550A>T	K139X
Case 5	Yes	G/C;A/T	1118C>T;1157T>C	No	wt	–
			1781G>A;1940G>A			
Case 6	NT	G/C	wt	–	wt	–
Case 7	No	G/C;A/T	wt	–	wt	–
Case 8	No	G/C;A/T	wt	–	wt	–
Case 9	No	G/C;A/T	wt	–	wt	–
Case 10	No	G/C;A/T	wt	–	wt	–

[a] 1pΔ, deletion in the short arm of chromosome 1 determined by fluorescence in situ hybridization with the telomeric D1Z2 probe performed on chromosome metaphases or interphase nuclei. NT: not tested. [b] Refers to the polymorphism of G/C and A/T alleles in exon 2, as determined by RT-PCR on mRNA and subsequent sequencing of products. [c] UISO carries an insertion on 1p36.2 of unknown origin (Van Gele et al, 1998). [d] NA, not available due to lack of *TP53* mRNA expression, as determined by RT-PCR. [e]Refers to the tightly linked polymorphisms located in exon 9 and exon 14, as determined by RT-PCR on mRNA and subsequent sequencing of products.

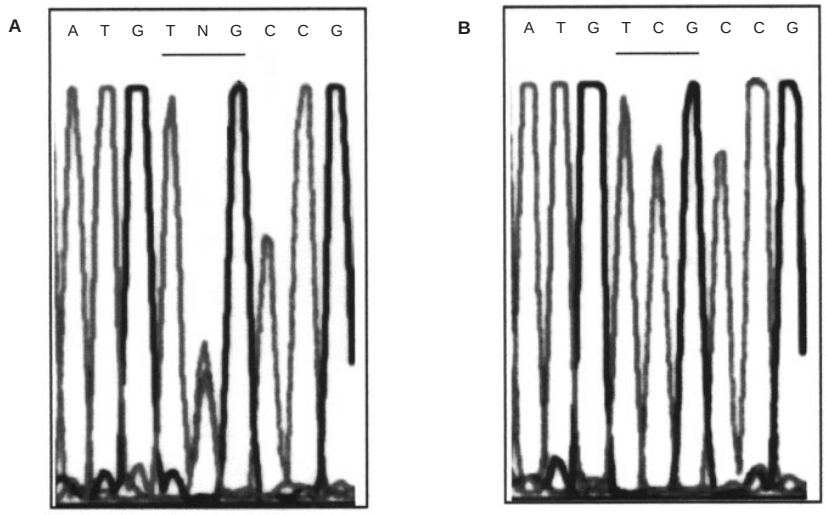

Figure 1 *p73* sequencing profiles of genomic tumour DNA (case 4) showing the heterozygous mutation at codon 110 (TCG→TTG) (**A**) and of the constitutional DNA (**B**)

RESULTS

Mutation analysis of *p73* and *TP53* in MCCs

cDNA of five MCC cell lines and ten MCC tumours was amplified by RT-PCR and, consequently, cycle-sequenced using specific nested PCR primers for the detection of mutations in *p73*. In addition, allelic expression for *p73* was determined based upon the known linked polymorphisms in exon 2. The sequencing results and allelic expression data are summarized in Table 1. A C to T mutation at codon 110 was found in one out of ten MCC tumours for *p73*, resulting in a serine to leucine substitution. This nucleotide exchange was also present in the genomic DNA of the tumour but not in the constitutional DNA (Figure 1). No mutations were found in the cell lines. Silent nucleotide substitutions at codon 336 (GCC to GCT) and codon 349 (CAT to CAC) located in exon 9 and at codon 557 (GCG to GCA) and at codon 610 (GCG to GCA) located in exon 14 were found in four (40%) of the ten examined tumours. These polymorphisms were also found in the constitutional patient DNA.

The same panel of MCC cell lines and MCC tumours was also examined for mutations in the *TP53* gene. Four *TP53* mutations were found in two out of five MCC cell lines and one non-sense mutation was observed in one MCC tumour (see Table 1).

DISCUSSION

Thus far, mutation analyses for *p73* have been performed in several types of carcinomas, oligodendrogliomas and neuroblastomas (Kovalev et al, 1998; Mai et al, 1998*a*, Nimura et al, 1998; Nomoto et al, 1998; Sunahara et al, 1998; Takahashi et al, 1998; Ichimiya et al 1999; Mihara et al, 1999). No mutations were found except for one somatic and one germline mutation in a series of 140 neuroblastomas (Ichimiya et al, 1999). Like MCC, neuroblastoma is a tumour of neuroendocrine origin frequently carrying distal 1p deletions. The two mutations found in neuroblastoma (P405R and P425L) were located within the COOH-terminal region of *p73* (Ichimiya et al, 1999). Recently, Takada et al (1999) showed that the COOH terminus of *p73* had a transactivation function whose activity was significantly reduced by the mis-sense mutations detected in the neuroblastoma tumours.

In the present study, one mis-sense mutation of *p73* in one out of ten MCC tumours was found: a heterozygous C to T transition at nucleotide 439, resulting in a serine to leucine substitution at codon 110 within exon 4. In comparison to the mutations observed in neuroblastoma, this mutation was located in the NH_2-terminal region (residues 1–112) of *p73*. The transcriptional transactivation function of the NH_2-terminal region of *p73* was shown to have an activity as strong as that of *TP53* (Takada et al, 1999). Further analysis may show that the observed mis-sense mutation in MCC leads to a reduction or complete loss of the transactivation function of the NH_2-terminal region of *p73*. The MCC tumour carrying the mutated *p73* allele still expresses the remaining wild-type allele. Similar to *TP53*, this mis-sense mutation might exert a dominant negative effect by an increased stability of the mutant protein compared to that of wild-type protein. Further studies are needed to verify this hypothesis.

The finding of *p73* mutations in neuroblastoma and MCC may also stimulate mutation analyses in other tumour types. Although the low frequency of mutations in both tumours seems to exclude a direct role for *p73* in tumorigenesis, other mechanisms than mutations in *p73* may lead to tissue-specific up- or down-regulation of the gene. In this respect, the available data on allelic expression of *p73* are conflicting. Nomoto et al (1998) found biallelic expression of *p73* in normal lung tissue and distinct patterns of allelic expression in different normal tissues. In contrast, Mai et al (1998*b*) reported monoallelic expression in normal lung tissue and increased and biallelic expression in lung tumour samples. Ichimiya et al (1999) found low *p73* expression in neuroblastoma, while *p73* transcripts were easily detectable in breast carcinoma and colorectal carcinoma under the same conditions. Although we performed no quantification of the *p73* mRNA, we observed biallelic expression in one MCC cell line and eight MCC primary tumours. Clearly, further studies on *p73* expression in normal and cancerous tissues is warranted.

As yet, only one study investigated the possible role of *TP53* in MCC (Schmid et al, 1997). Using immunohistochemical staining, TP53 expression was studied in 25 Merkel cell carcinomas. Only five tumours showed 5–10% TP53-immunoreactive tumour nuclei. Single-strand conformation polymorphism (SSCP) analysis

for exons 4–8 did not reveal mutations in these five tumours which lead the authors to conclude that *TP53* alterations play only a minor part in the genesis of MCC. However, using this approach it could not be ruled out that the presence of mutations in the other exons of *TP53* were missed. Also, mis-sense and non-sense mutations not leading to accumulation of the gene product would be overlooked in those cases not investigated by SSCP analysis. We also investigated *TP53* for the presence of mutations by sequencing of the entire coding region of the *TP53* gene. *TP53* mutations were found in two out of five MCC cell lines and in one out of ten tumours. In cell line MCC13, three mutations were found at dipyrimidine sites, namely a double base pair C to T transition at nucleotides 857, 858 and a single base pair C to T transition at nucleotide 967 both leading to amino acid substitutions at codon 241 and codon 278 respectively (Table 1). These type of mutations are characteristically induced by absorption of UV irradiation by the DNA (Brash et al, 1991). The mutation found in MCC14/2 is a hemizygous T to A transversion at nucleotide 950 since no wild-type sequence was present. This point mutation is translated into an amino acid substitution from valine to glutamic acid at codon 272. The amino acid changes at codon 241, 272 and 278 reside in mutational hotspots of the sequence-specific DNA-binding domain of the TP53 protein. Alterations in these residues of the TP53 protein will typically result in defective contacts with the DNA and finally lead to the loss of the ability of TP53 to act as a transcription factor. For the particular amino acid substitution at residue 241, Cho et al (1994) could show that DNA contact was not possible as the defective TP53 protein was unable to make contact with the phosphate backbone in the major groove of the DNA. The non-sense mutation at nucleotide 550 in the MCC tumour (case 4) predicted the generation of a truncated TP53 protein. Accumulation of truncated TP53 variants can result in a dominant negative effect and loss of normal TP53 function.

In conclusion, we describe the finding of a sporadic *p73* NH_2-terminal located mis-sense mutation in MCC together with the observation of two previously reported loss of function mutations within the COOH-terminal transactivation region of *p73* in neuroblastoma. These mutations provide circumstantial evidence for a role of *p73* in these malignancies of neuroectodermal origin. At present, the exact physiological function of *p73* in tumour development is unclear. In view of these findings, we propose further investigations of *p73* in normal development and tumorigenesis. Further insights in its role in apoptosis (Jost et al, 1997) and possibly other mechanisms such as cell cycle control and cell differentation may provide additional clues for possible contribution of *p73* in tumorigenesis. This report also shows for the first time the occurrence of inactivating *TP53* mutations in 19% of the investigated MCCs. The C to T mutations in cell line MCC13 are known to be caused by UVB irradiation and thus provide further evidence for sun-exposure in some Merkel cell carcinomas.

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3.1.2 Frequent allelic loss at 10q23 but low incidence of *PTEN* mutations in Merkel cell carcinoma

The finding of loss of chromosome 10 or the chromosome 10 long arm in more than one third of the studied MCC cases by CGH led us to assume that loss of gene function of a putative tumor suppressor gene located on 10q could contribute to MCC development or progression. In 1997, a new tumor suppressor gene called *PTEN* (alias *MMAC1* or *TEP1*) was identified and mapped onto 10q23.3. *PTEN* encodes for a dual-specificity phosphatase and shows extensive homology with the cytoskeletal proteins tensin and auxilin which are involved in focal cell adhesion (Li and Sun, 1997; Li *et al.*, 1997; Steck *et al.*, 1997). Studies with *Pten* knockout mice showed that *Pten* is essential for embryonic development and tumor suppression through its ability to control cellular differentiation and anchorage-dependent growth (Di Cristofano *et al.*, 1998). In addition, *PTEN* is involved in cell proliferation and regulation of apoptosis through negative regulation of the *PI3K/PKB/Akt* cell survival signaling pathway (see Figure 3-2) (Stambolic *et al.*, 1998; Yamada and Araki, 2001). *PTEN* is frequently mutated in a wide variety of human cancers including brain, breast, endometrium, kidney and prostate, indicating its critical role in the pathogenesis of many neoplasms. A very high proportion of *PTEN* mutations are located in the phosphatase core motif located in exon 5, pointing at the importance of the phosphatase activity in the tumor suppressor function of *PTEN* (Ali *et al.*, 1999).

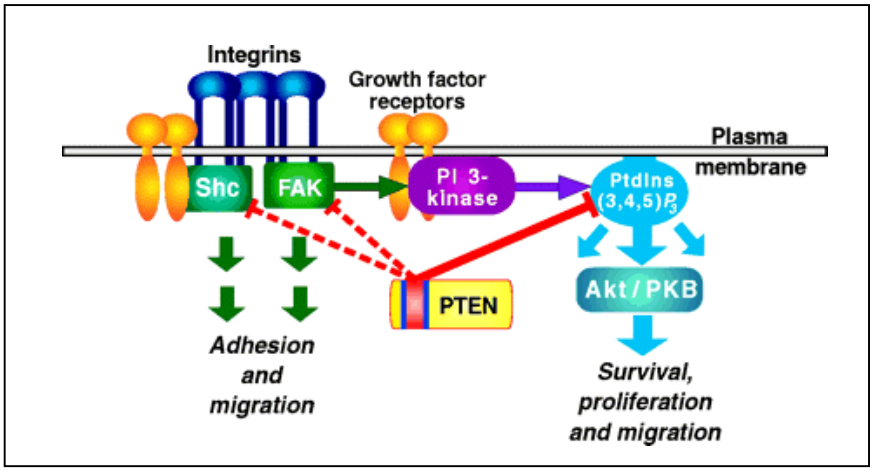

Figure 3-2: Reported sites of action of PTEN. Extracellular interactions trigger signaling from integrins and growth factor receptors. The major function of PTEN is downregulation of the PI3-kinase product PtdIns(3,4,5)P_3, which regulates Akt and complex downstream pathways affecting cell growth, survival and migration. In addition, PTEN has weak protein tyrosine phosphatase activity, which may target focal adhesion kinase (FAK) and Shc, and thereby modulate other complex pathways. The phosphatase domain of PTEN (red) dephosphorylates and downregulates (red lines) substrate molecules.

(Reproduced from Yamada and Araki, J Cell Science, 114: 2375-2382, 2001)

In view of the above findings, we decided to investigate the role of the *PTEN* gene in MCC. In keeping with our CGH data, LOH analysis revealed a high frequency of loss at the *PTEN* locus (43% of informative cases). Despite this high incidence of LOH only one homozygous deletion for exon 9 and one non-sense mutation at exon 5, respectively were detected. The latter was located in the phosphatase domain of *PTEN* and could result in a reduced or complete loss of the tumor suppressor function of *PTEN*. Our results suggest that loss of one *PTEN* allele and disruption of the second allele by mutation and homozygous deletion is a relatively rare event in MCC. We propose that other alternative mechanisms can lead to inactivation of *PTEN* or that other tumor suppressor genes at chromosome 10 are implicated in the development of MCC.

Recently, studies showed that the loss of one *PTEN* allele (haploinsufficiency) is sufficient to induce tumor formation and progression in certain cancer types (Kwabi-Addo *et al.*, 2001). We assume that this mechanism can also be implicated in MCC (see General Discussion).

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FREQUENT ALLELIC LOSS AT 10Q23 BUT LOW INCIDENCE OF *PTEN* MUTATIONS IN MERKEL CELL CARCINOMA

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Merkel cell carcinoma (MCC) is a rare, highly metastatic skin tumor of neuroectodermal origin. The disease shares clinical and histopathological features with small cell lung carcinoma (SCLC). The genetic mechanisms underlying the development and tumor progression of MCC are poorly understood. We recently showed by comparative genomic hybridization (CGH) that the pattern of chromosomal abnormalities in MCC resembles that of SCLC. One of the most frequently observed losses involved the entire chromosome 10 or partial loss of the chromosome 10 long arm (33% of examined MCC cases). The *PTEN* tumor-suppressor gene has been mapped to 10q23.3 and was shown to be mutated in a variety of human cancers including SCLC. Germline *PTEN* mutations have been observed in familial predisposing cancer syndromes including Cowden disease. Interestingly, an association between Cowden syndrome and Merkel cell carcinoma has been reported. To study the possible role of *PTEN* in MCC oncogenesis, loss of heterozygosity (LOH) analysis for the 10q23 region was performed on 26 MCC tumor samples from 23 MCC patients. The *PTEN* locus was deleted in 9 of 21 (43%) informative MCC tumor samples [7 of 18 (39%) MCC patients]. Despite this high frequency of LOH at 10q23, mutation and homozygous deletion screening of the *PTEN* gene revealed only one tumor with a nonsense mutation and a second with a homozygous deletion of exon 9. These data suggest that either alternative mechanisms lead to inactivation of the *PTEN* gene or that other tumor-suppressor genes at chromosome 10 are implicated in the development of MCC.

Key words: *PTEN; Merkel cell carcinoma; loss of heterozygosity; nonsense mutation; homozygous deletion*

Merkel cell carcinoma (MCC) is a rare neuroendocrine skin tumor with aggressive clinical behavior. Tumors occur mostly in the head and neck region or on the arms in older, fair-skinned people, implicating UV exposure in the etiology.[1] The tumor shares common features with small cell lung carcinoma (SCLC), including morphological and immunophenotypical characteristics.[2] Both are small cell tumor types with neuroendocrine features expressing specific neuropeptides and intermediate filament proteins.[3] Similarities in clinical behavior include a high incidence of recurrences and nodal metastases.[4]

Little is known about the genetic background of Merkel cell carcinoma. Although cytogenetic findings have been described in about 30 MCCs, no recurrent chromosomal abnormalities have been identified except for loss of distal 1p material due to various types of chromosomal aberrations. Further support for 1p involvement in MCC was provided by Van Gele *et al.*,[5] who showed that 2 distinct regions on 1p36 are implicated in MCC. In the search for chromosomal losses indicating possible involvement of tumor-suppressor genes in MCC, loss of heterozygosity (LOH) studies for markers on 1p, 3p and 13q have been undertaken.[6–9] A genome-wide analysis of chromosomal losses and gains was performed by comparative genomic hybridization (CGH) of 26 MCC tumors and 8 MCC cell lines. This study revealed a characteristic pattern of gains and losses in Merkel cell carcinoma pointing at regions harboring putative oncogenes and tumor-suppressor genes.[10] Interestingly, the CGH pattern of MCC closely resembles the pattern of chromosomal gains and losses observed in SCLC.[11] One of the more frequently observed losses in MCC involved the entire chromosome 10 or partial loss of the chromosome 10 long arm.[10] Chromosome band 10q23.3 harbors the *PTEN* tumor-suppressor gene for which mutations and deletions have been reported in a variety of human neoplasms including SCLC.[12]

Germline mutations of *PTEN* predispose affected individuals to develop various benign and malignant lesions in a wide spectrum of tissues. These cancer-predisposing syndromes include Bannayan-Zonana syndrome, Juvenile Polyposis Coli and Cowden syndrome.[13–15] Interestingly, Haibach *et al.*[16] reported a patient with Cowden syndrome who developed renal cell carcinoma and Merkel cell carcinoma. Based upon the above observations, we hypothesised that *PTEN* might play a role in the development or progression of highly metastatic Merkel cell carcinomas. To investigate this, LOH analysis at chromosomal band 10q23 and mutational analysis of *PTEN* was performed on 26 MCC tumor samples representing 23 patients and 8 MCC cell lines. In addition, all samples were examined for the presence of intragenic homozygous *PTEN* deletions.

MATERIAL AND METHODS

Tumor specimens and cell lines

Tumor and peripheral blood samples were obtained at the Queensland Radium Institute Laboratory, Queensland Institute of Medical Research, Brisbane, Australia, and at the Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium. Clinical data of all patients are summarized in Table I. LOH and CGH data for these tumors have been reported previously.[10,17] Morphological characterization and cytogenetic data of the MCC cell lines have been published previously[18–21] except for MCC26 and T95-45, which will be published elsewhere (Van Gele *et al.*, in preparation). Genomic DNA was extracted from cell lines and frozen sections of tumor samples by using standard proteinase-K digestion, phenol/chloroform extraction and ethanol precipitation protocols. Constitutional DNA was isolated from peripheral blood lymphocytes using the Qiagen blood and cell culture DNA Midi kit (Qiagen, Hilden, Germany). The investigated tumor samples contained at least 60% tumor cells.

LOH analysis

Four microsatellite markers, known to map to the 10q23 region, were used for LOH analysis. The order of these markers from centromere to telomere is D10S1687, D10S215, *PTENCA* and D10S541 and was based on the genetic map of chromosome 10q (http://www-genome.wi.mit.edu). Primer sequences were obtained from the Genome Database except for *PTENCA.*[22] PCR amplification was performed in a 25 μl reaction volume using 200 ng of

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genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM each dNTP, 30 mM of each primer and 1 U Platinum Taq DNA polymerase (Life Technologies, Merelbeke, Belgium) on an MJ Research PTC200 thermal cycler. Cycling conditions were 92°C for 3 min, followed by 35 cycles at 92°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, with a final extension period of 72°C for 7 min. Each forward primer defining a microsatellite repeat marker was 5′-tagged with the fluorescent dye labels HEX, TET or 6-FAM (Biosource International, Keystone Laboratories, CA). PCR products were electrophoresed on 6% LongRanger (FMC Bioproducts, ME) 6 M urea denaturing gels using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Lennik, Belgium) and the GeneScan 500 [TAMRA] internal size standard (Applied Biosystems). Allele size and fluorescent intensity were determined by GeneScan Analysis 2.1 software (Applied Biosystems). LOH was analysed by determining the fluorescent intensity of each allele and calculating the ratio of peak intensities. A sample was scored as having LOH if an allelic ratio of ≤ 0.5 or ≥ 2.0 was obtained.

PTEN *mutation analysis*

Intronic primers flanking each of the 9 exons of the *PTEN* gene were used for amplification of tumor DNA. Primer sequences were described by Steck *et al.*,[23] except for exon 5 for which new primers were designed using Primer Express Software version 1.0 (Applied Biosystems): Ex5 forward (5′TTTTCTCTGGAATCCAGTGTTTCT3′) and Ex5 reverse (5′AGATCCAGGAAGAGGAAAGGAA3′). PCR amplification was performed in a 25 μl reaction volume using 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM each dNTP, 30 mM of each primer and 1 U Platinum Taq DNA polymerase (Gibco BRL) on an MJ Research PTC200 thermal cycler. Cycling conditions were 92°C for 3 min, followed by 35 cycles at 92°C for 30 sec, 53°C for 30 sec and 72°C for 1 min, with a final extension period of 72°C for 7 min. A different annealing temperature was used for exon 5 (60°C). PCR products were purified by using the Qiaquick PCR purification kit (Qiagen). Subsequently, the PCR products were cycle sequenced on an ABI PRISM 377 sequencer according to the manufacturer's instructions (Applied Biosystems). The same primers (forward and reverse) as those for amplification of tumor DNA were used for the sequencing reactions. Nucleotide changes were confirmed by reamplification and resequencing of tumor DNA and corresponding constitutional DNA.

Screening for homozygous PTEN *deletions*

A multiplex PCR assay was used to screen for homozygous deletions in the *PTEN* gene. Each *PTEN* exon (same primers as above) was co-amplified with a 983 bp control fragment encoding the human GAPDH gene (primers from Clontech, Heidelberg, Germany). The same PCR cycling conditions were used as for the *PTEN* mutation analysis except that multiplex PCR was performed at 30 cycles for the MCC cell lines and at 25 cycles for all tumor samples and their corresponding constitutional DNA. PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide, and signal strengths were compared and interpreted by 2 independent persons.

TABLE I – CLINICAL DATA FROM 26 SAMPLES OF 23 PATIENTS WITH MCC

Tumor	Sex	Age at presentation	Specimen[1]
MCC2	M	66	Node
MCC3	M	68	Node
MCC4	M	76	Node
MCC5	F	72	Rec
MCC7T1/T2[2]	F	63	Rec/Met
MCC8	M	84	Rec
MCC9	M	81	Rec
MCC11	M	71	Rec
MCC12	M	60	Rec
MCC13	F	65	Rec
MCC14T1/T2	M	80	Node/Node
MCC16	M	70	Rec
MCC17	M	74	Rec
MCC18	M	64	Rec
MCC21	M	53	Node
MCC22	M	72	Node
MCC24	M	79	Rec
MCC26T1/T2	F	82	Rec/Node
UHG-VM	F	79	Met
UHG-RM	F	62	Met
UGH-FA	F	69	Prim
RJ	M	86	Prim
SA	F	66	Prim

[1]Node, specimen taken from regional lymph node; Rec, recurrence at site; Met, metastasis; Prim, primary tumor.–[2]T1, first tumor specimen; T2, second tumor specimen.

RESULTS

LOH analysis

A panel of 26 MCC tumor samples from 23 patients and corresponding constitutional DNAs (except for MCC21 and UHG-RM) were analysed using 3 microsatellite markers flanking the *PTEN* locus (D10S1687, D10S215 and D10S541) and marker *PTENCA*, which is located in the *PTEN* gene. Results of LOH analyses are summarized in Table II. The frequencies of LOH for the 4 loci on chromosome 10q23 were 6 of 20 informative tumor samples (30%) [5 of 19 patients (26%)] at D10S1687, 3 of 9 informative tumor samples (33%) [3 of 9 patients (33%)] at D10S215, 9 of 21 informative tumor samples (43%) [7 of 18 patients (39%)] at *PTENCA* and 7 of 17 informative tumor samples (41%) [6 of 16 patients (37.5%)] at D10S541. LOH at one or more loci was detected in 10 of the 26 MCC tumor samples examined (38%), representing 8 of the 23 patients (35%) (MCC2, 8, 11, 12, 14, 18, 26 and UHG-RJ). *PTENCA* was the most frequently affected marker in these tumor samples.

TABLE II – RESULTS OF LOH ANALYSIS AT THE 10q23 REGION IN 23 PATIENTS WITH MCC

Marker cen → tel	D10S1687	D10S215	PTENCA	D10S541
MCC2	LOH	H	LOH	LOH
MCC3	ROH	H	ROH	ROH
MCC4	ROH	H	ROH	ROH
MCC5	H	H	ROH	H
MCC7T1	ROH	H	ROH	H
MCC7T2	ROH	H	ROH	H
MCC8	H	LOH	H	LOH
MCC9	ROH	ROH	ROH	ROH
MCC11	LOH	LOH	LOH	LOH
MCC12	H	H	LOH	LOH
MCC13	ROH	ROH	ROH	ROH
MCC14T1	LOH	H	LOH	LOH
MCC14T2	LOH	H	LOH	LOH
MCC16	ROH	ROH	H	ROH
MCC17	ROH	H	ROH	ROH
MCC18	LOH	LOH	LOH	LOH
MCC21	ROH	ROH	H	H
MCC22	ROH	H	ROH	ROH
MCC24	ROH	H	ROH	ROH
MCC26T1	H	NA	LOH	H
MCC26T2	H	NA	LOH	H
UHG-VM	ROH	ROH	ROH	H
UHG-SA	ROH	H	ROH	H
UHG-RM	H	H	H	ROH
UHG-FA	ROH	ROH	H	ROH
UHG-RJ	LOH	H	LOH	H

ROH, retention of heterozygosity; H, homozygosity (not informative); LOH, loss of heterozygosity; NA, not available due to no interpretable results; T1, T2, tumor specimens from same tumor but obtained from different sites.

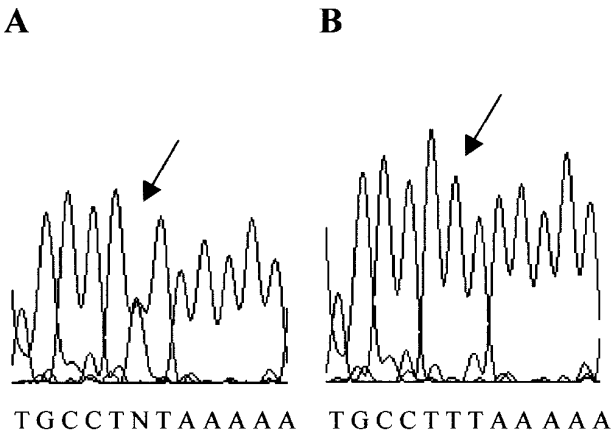

FIGURE 1 – A detail of the reverse sequencing profile of exon 5 of *PTEN* showing the point mutation in tumor MCC2 at codon 147 (*a*). Same sequencing profile but in the constitutional DNA of patient MCC2 (*b*).

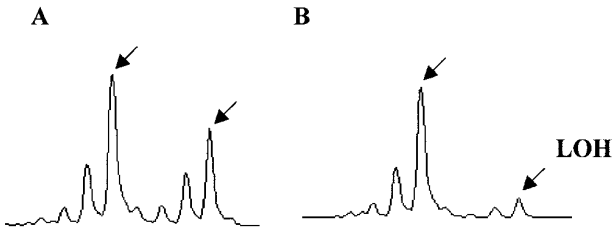

FIGURE 2 – Loss of heterozygosity analysis in tumor MCC2 for the polymorphic marker *PTENCA*. The alleles present in the constitutional DNA (*a*) and in the tumor DNA (*b*) of patient MCC2 are indicated by arrows. LOH, loss of heterozygosity.

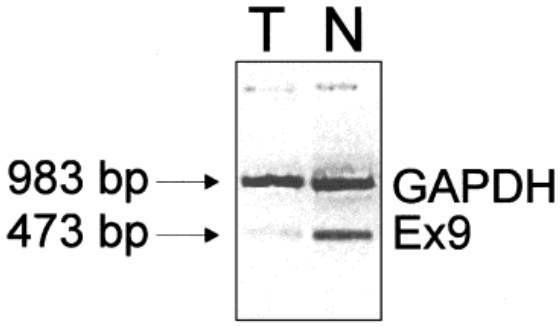

FIGURE 3 – Detail of multiplex PCR with GAPDH and exon 9 of the *PTEN* gene showing a homozygous deletion in tumor MCC5. PCR products were visualised on a 1.5% agarose gel. Sizes in base pairs are shown on the right. T, tumor DNA; N, constitutional DNA of patient MCC5.

Screening for PTEN *mutations and homozygous deletions in MCC tumors and cell lines*

All 26 MCC tumor samples and 8 additional MCC cell lines were analysed for mutations of the *PTEN* gene by direct sequencing of all 9 *PTEN* exons. Two polymorphisms at position IVS8 +32 (G/T) and IVS2 –96 (G/A) were found in most of the examined samples (58.8% and 82.3%, respectively). A nonsense mutation was detected at exon 5 in 1 MCC tumor (MCC2), leading to an AAG to TAG change at codon 147, which was not seen in the constitutional DNA (Fig. 1*a*,*b*). This mutation would probably result in a premature termination of the PTEN protein at the same codon leading to a complete loss of PTEN protein activity. To our knowledge, this *PTEN* mutation has not been reported before. The same tumor also showed loss for all informative loci including the *PTENCA* marker (Fig. 2). Despite loss of 1 *PTEN* allele, the sequencing profile of the mutation shows a heterozygous mutation (Fig. 1*a*). This observation could be the result of genetic heterogeneity within the tumor biopsy as evidenced by presence of mitoses with and without monosomy for chromosome 10.[17] CGH clearly demonstrated loss of the 10q region in this tumor.[10]

In addition, all samples were screened for homozygous *PTEN* deletions by multiplex PCR. Due to the presence of specific PCR products for all 9 *PTEN* exons in all MCC cell lines, we assumed them not to carry homozygous *PTEN* deletions. In 1 MCC tumor (MCC5), the PCR assay showed a reduced PCR product for exon 9 when compared with the corresponding constitutional DNA, in contrast to the equal signals for GAPDH, strongly suggesting the presence of a homozygous deletion for this particular exon (Fig. 3). In the tumor DNA, a faint band is still present, probably due to stromal contamination within the tumor specimen.

DISCUSSION

The *PTEN* gene (also called *MMAC1* or *TEP1*) encodes for a dual-specificity protein phosphatase that can dephosphorylate tyrosine, serine and threonine residues. *PTEN* also shows extensive homology with the cytoskeletal proteins tensin and auxilin which are involved in focal cell adhesion.[23–25] Recent studies with *PTEN* knockout mice showed that *PTEN* is essential for embryonic development and tumor suppression through its ability to control cellular differentiation and anchorage-dependent growth.[26] Moreover, *PTEN* seems to be involved in cell proliferation and regulation of apoptosis through negative regulation of the *PI3′K/PKB/Akt* cell survival signaling pathway.[27] A high frequency of reported *PTEN* mutations resides within the highly conserved protein tyrosine phosphatase (PTPase) core motif located in exon 5 at residues 122–132 (IHCKAGKGRTG).[14] This points to the importance of the phosphatase activity in the tumor-suppression function of *PTEN*. Genetic changes of *PTEN* occur in multiple cancer types, suggesting that *PTEN* may play a more general role in the pathogenesis of a variety of human malignancies. The following observations prompted us to perform LOH analysis of the 10q23 region and to search for inactivating *PTEN* mutations and homozygous deletions in MCC: 1. reported CGH data showing loss of chromosome 10 or the long arm of chromosome 10 in 33% of examined cases;[10] 2. clinical and histopathological resemblance between MCC and SCLC and reported deletions and mutations of the *PTEN* gene in SCLC;[2–4,28,29] 3. the finding of an MCC in a patient with Cowden disease, a syndrome for which germline *PTEN* mutations have been reported.[16] LOH of 1 or more loci at the 10q23 region was detected in 38% (10 of 26) of the examined MCC tumor samples [8 of 23 (35%) MCC patients]. The *PTEN* locus was deleted in 9 of 21 (43%) informative samples [7 of 18 (39%) MCC patients]. Despite the high incidence of LOH at the *PTEN* locus, *PTEN* mutations were detected in only 1 tumor of 34 examined MCCs. The detected somatic point mutation, resulting in a change from lysine to a stop codon, was located in the phosphatase domain of *PTEN*, 15 codons downstream of the PTPase core motif. This nonsense mutation might lead to a truncated protein missing a part of exon 5 and all the following exons (6, 7, 8 and 9), resulting in complete loss of PTEN protein activity or a truncated protein with reduced phosphatase activity leading to a decrease or inactivation of the tumor-suppressor function of *PTEN*. Alternatively, loss of the C-terminal region of *PTEN* could lead to loss of a particularly important but as yet undefined function, as the role of the C-terminus remains unclear. In addition, exon 9 of *PTEN* was found to be homozygously deleted in 1 MCC tumor. Unfortunately, no RNA or cell lysates of these tumors were available to examine the above mentioned alterations in further detail.

Overall, our data suggest that inactivation of *PTEN* by loss of 1 allele and disruption of the second allele by mutation or homozygous deletion is a relatively rare event in the pathogenesis of MCC. Other mechanisms leading to deregulation or inactivation of *PTEN* may be involved. For example, down-regulation at the mRNA or protein level of PTEN has been reported for prostate cancer in at least 50% of advanced human prostate cancers.[30] A report by Zhou *et al.*[31] suggested an epigenetic mechanism of bi-allelic functional inactivation of *PTEN* in malignant melanomas without *PTEN* mutations or deletions. Mutations located in the promoter region or in intronic sequences may result in *PTEN* inactivation. Alternatively, 1 or more unidentified tumor-suppressor genes within the 10q23 deleted region could also be involved in the oncogenesis of Merkel cell carcinoma.

Since CGH indicated loss of an entire copy of chromosome 10 in one-quarter of the examined MCC patients,[10] we cannot exclude that 1 or more other tumor-suppressor genes located either on 10p or 10q might be involved in the etiology of MCC. Several putative tumor-suppressor genes have been mapped to 10q. In the *DMBT1* gene, intragenic homozygous deletions were detected in medulloblastomas and glioblastoma multiforme.[32] A study by Wu *et al.*[33] showed that in contrast to high expression of DMBT1 in normal adult lung tissue, DMBT1 expression was absent in 100% of SCLC cell lines and 43% of NSCLC cell lines due to gene deletion and to other yet unidentified mechanisms. *MXI1* is mutated in a small fraction of prostate carcinomas.[34] The *LGI1* gene, predominantly expressed in neural tissue, showed reduced expression in low-grade brain tumors and significantly reduced or absent expression in malignant gliomas.[35] The *h-neu* gene was expressed at very low level in human malignant astrocytoma tissues and the majority of examined glioma cell lines, while normal brain expressed higher levels of *h-neu* transcripts. One of the glioma cell lines had a single nucleotide substitution. It is hypothesized that the *h-neu* gene plays a role in determination of cell fate in the human central nervous system, and it is conceivable that a loss of function of the *h-neu* gene may lead to the development of neuroectodermal tumors.[36] Previous studies of human gliomas and primary glioblastomas showed, beside loss of 10q in these tumors, patterns of deletions on 10p14–15 possibly harbouring not yet identified putative tumor-suppressor gene(s).[37–41] Analysis of additional Merkel cell carcinomas and detection of small deletions on 10p and/or 10q may allow further refined delineation and molecular analysis of the most likely candidate tumor-suppressor genes within these regions.

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PART 4

Differential Gene Expression Analysis

4.1 Differential gene expression analysis in Merkel cell and small cell lung carcinoma

4.1.1 Analysis of gene expression profiles in Merkel cell and small cell lung carcinoma: improved differential diagnosis and classification of different Merkel cell phenotypes *Van Gele Mireille et al. (in preparation)*

4.1.1 Analysis of gene expression profiles in Merkel cell and small cell lung carcinoma: improved differential diagnosis and classification of different Merkel cell phenotypes

At present few insights into the genetic defects and molecular pathways involved in MCC pathogenesis are available. The genome wide screening approach described in Paper 2.1.2 identified new regions of recurrent gains and losses involved in MCC and revealed a number of common genetic changes for MCC and SCLC (overviewed in Figure 4-1) indicating that certain biological pathways or genes are implicated in the etiology of both tumors. Evidence for frequent inactivation of tumor suppressor genes such as *FHIT*, *RB1*, and *TP53* was obtained in SCLC whereas *PTEN*, *TP73* and *P16*^{INK4a} seemed rarely involved in this tumor type (Minna *et al.*, 2002). Molecular studies described in this thesis (see Part 3) and reported by other groups (Sozzi *et al.*, 1996a; Leonard and Hayward, 1997; Cook *et al.*, 2001) revealed that the same genes were also involved in MCC.

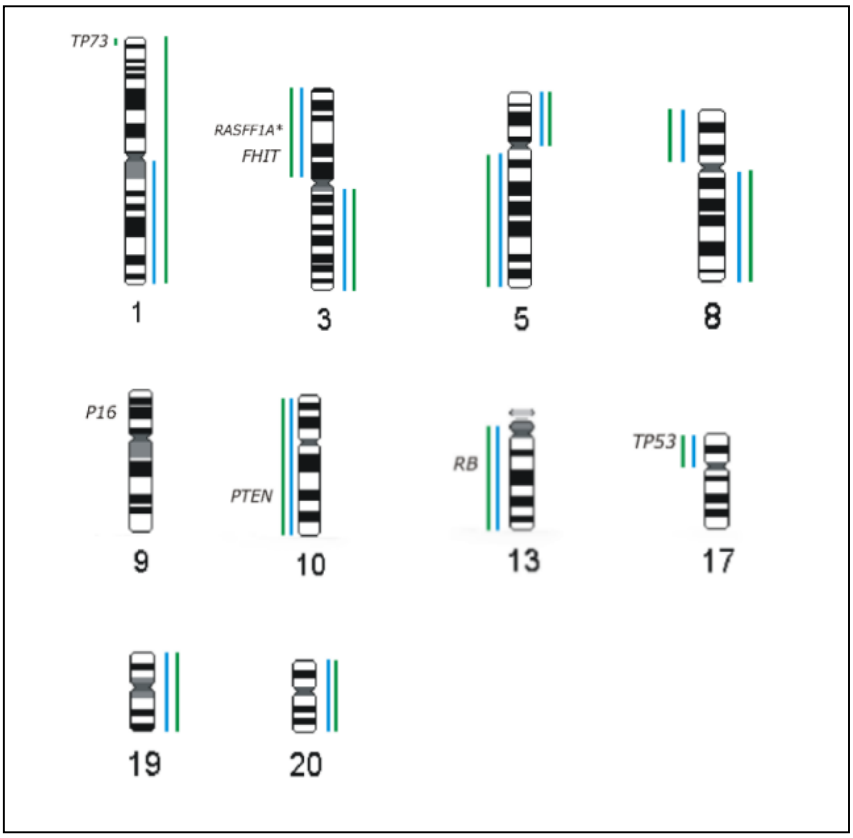

Figure 4-1: Overview of common recurrent gains and losses between Merkel cell (MCC, green bars) and small cell lung carcinoma (SCLC, blue bars). Lines on the right side of the ideograms show overrepresentations, lines on the left side show underrepresentations. Tumor suppressor genes investigated in both SCLC and MCC are indicated next to the ideograms (*only examined in SCLC).

Besides these similarities in occurrence of genetic alterations in MCC and SCLC, both tumors also share other features such as (a) small round blue cell histology; (b) presence of neurosecretory granules; (c) neuroendocrine origin; (d) expression of identical histochemical markers; (e) clinical aggressive behavior and (f) variable response to therapy. These similarities may hamper the differential diagnosis for these tumors, especially when no obvious primary lesion can be detected. Identification of additional molecular markers could thus improve the differential diagnosis between both tumors. Cell lines derived from both tumors also showed identical morphological and growth characteristics. Analogous to SCLC, MCC cell lines were classified into two phenotypically different subgroups, i.e. Classic and Variant types (Carney *et al.*, 1985; Gazdar *et al.*, 1985; Leonard and Bell, 1997). Previous studies showed that SCLC tumors from whom Variant SCLC cell lines were established behaved aggressively, patients responded poorly to therapy and showed a worse prognosis compared to those tumors from whom Classic SCLC cell lines were derived, indicating biological and clinical differences between both groups (Gazdar *et al.*, 1985). A number of histochemical markers (Broers *et al.*, 1985; Broers *et al.*, 1986) and recently also genes differentially expressed between Classic and Variant SCLC cell lines (Zhang *et al.*, 2000), have been identified thus offering insight into the molecular basis of their different malignant behavior. In contrast to SCLC, no differential gene expression studies have been performed for MCC. Only one recent molecular study indicated that lack of the *Brn-3c* transcription factor and/or basic helix-loop-helix transcription factor *HATH1* was correlated with loss of the neuroendocrine phenotype in Variant MCC cell lines and could serve as useful prognostic markers (Leonard *et al.*, in press). Although this study yielded valuable information, it is obvious that additional studies are warranted to gain more insight into the biology of MCC.

In order to learn more about the molecular pathogenesis of MCC and identify molecular markers which could improve the differential diagnosis between MCC and SCLC, we determined the gene expression profiles of 10 MCC and 4 SCLC cell lines using Atlas cDNA filter expression arrays containing 1891 unique genes involved in many cellular processes. Supervised analysis of the data allowed us to identify a set of 23 classifier genes which could improve differential diagnosis between MCC and SCLC. Some of these molecular markers or disrupted pathways could be potential targets for new therapeutic treatments and improve patient survival in MCC and/or SCLC. In addition, we identified

89 significant differentially expressed genes which allowed classification of the MCC cell lines into their Variant and Classic subgroups. We assume that the differential expression levels of some of these genes reflect, analogous to SCLC, the different biological and clinical properties of MCC phenotypes. This would implicate a more aggressive treatment for tumors with a Variant morphology and a closer follow-up for this patient group.

Analysis of gene expression profiles in Merkel cell and small cell lung carcinoma: improved differential diagnosis and classification of different Merkel cell phenotypes

Van Gele Mireille, *et al*. (in preparation)

Introduction

Merkel cell carcinoma (MCC) is a rare aggressive neuroendocrine skin tumor, mostly affecting elderly people. The disease occurs predominantly on the sun-exposed areas of the skin suggesting UV-exposure in its etiology (reviewed in[1]). The number of MCC tumors and cell lines which have been cytogenetically investigated is relatively small[2]. Although no recurrent translocations have been reported, cytogenetic analysis showed near-diploid karyotypes with complex structural changes in most cases, often presenting as multi-way translocations. The distal part of the short arm of chromosome 1 is frequently affected and has been reported as the sole structural chromosome change in 3 MCC tumors[3, 4] (Van Gele *et al*., unpublished results). In contrast to the rather inhomogeneous pattern of chromosomal changes described in cytogenetic studies, CGH analysis revealed a number of typical chromosomal imbalances including loss of chromosomal regions 3p, 5q, 8p, 10, 11q, 13q and 17p and gain of chromosomal regions 1, 3q, 5p and 8q[5]. Interestingly, the pattern of gains and losses resembled the pattern observed in previous CGH reports of small cell lung carcinoma (SCLC)[6-8].

MCC and SCLC are both derived from neuroendocrine cells (Merkel and Kulchitsky cells, respectively) expressing peptide hormones such as neuron-specific enolase and bombesin. Histologically, both tumors are composed of small round blue cells and contain often neurosecretory granules visible by electron microscopy. Clinically, they are aggressive tumors responding initially to chemotherapy, but response is typically short-lived. They show a high degree of local recurrence and have a tendency to metastazise early[9]. Differential diagnosis between MCC and SCLC is often difficult in SCLC patients presenting with metastasis to the skin especially when the primary lesion is not detected. Identification of additional molecular markers could thus improve/facilitate the differential diagnosis between both tumor types. Moreover, further insights into the genes and signaling pathways involved in these tumors is a prerequisite for development of the new rational therapeutic interventions which could lead to an improved patient survival or even complete remission.

In addition to the above mentioned clinical and histological resemblance between MCC and SCLC, similarities in morphology and growth characteristics were observed in cell lines derived from both tumors. Comparable to SCLC cell lines, MCC cell lines can also be grouped into four different morphological classes (Type I to IV). In addition, different expression patterns of cytokeratins and neuroendocrine markers, such as chromogranin A and neuron-specific enolase, allowed further classification of these cell lines into "Classic" and "Variant" groups[10, 11]. Recent studies on the POU-IV family member *Brn-3c* and basic helix-loop-helix transcription factor *HATH1* showed that lack of their expression was linked to loss of the neuroendocrine phenotype of Variant MCC cell lines[12].

In contrast to MCC, numerous molecular genetic studies have been performed on SCLC which contributed to the understanding of SCLC pathogenesis (reviewed in[13, 14]). In addition, histochemical markers and differentially expressed genes distinguishing Classic from Variant SCLC cell lines have been identified and led to an improved understanding of

the underlying genetic basis responsible for the biological and clinical heterogeneity among small cell lung cancers[15, 16].

In order to obtain further insights into the molecular pathogenesis of MCC, the factors influencing cell line morphology, aggressiveness and neuroendocrine phenotype and the relationship of MCC versus SCLC, we decided to determine the gene expression profiles of 10 MCC cell lines and four SCLC cell lines using Atlas cDNA expression arrays containing 1891 unique genes involved in many cellular functions.

Material and Methods

Cell lines

Merkel cell carcinoma cell lines MCC5, MCC6, MCC13, MCC14/1, MCC14/2 and MCC26 were established at the Queensland Radium Institute Laboratory, Queensland, Australia and have been described in detail previously by Leonard *et al*.[17, 18]. MCC cell line UISO was described by Ronan *et al*.[19], MKL-1 by Rosen *et al*.[20]. MKL-2 was established at the Robert H. Lurie Comprehensive Cancer Center, Illinois, USA and reported by Van Gele *et al*.[2]. T95-45 was established at the Center for Medical Genetics, Ghent, Belgium. Most cell lines were previously analyzed by CGH and/or M-FISH[2, 5]. Small cell lung carcinoma cell lines NCI-H69 and NCI-146 were obtained from the American Type Culture Collection, CorL88 was a gift from Dr. P. Twentyman, Cambridge, UK and GLC4 was a gift from Dr. Marc Maliepaard, Amsterdam, The Netherlands. Neuroblastoma cell lines IMR32[21], SK-N-SH[22], SK-N-AS[23], NGP[24] and N206[25] were included for analysis. All cell lines were grown to subconfluency in RPMI 1640 (Invitrogen) supplemented with antibiotics, 15% fetal calf serum and 1% L-glutamine. Cells from 10 tissue culture flasks (75 cm^2) were pelleted, quick frozen in liquid N_2 and stored at –80°C. Melanocytes and neonatal foreskin fibroblasts (NFF) were isolated at the Queensland Radium Institute Laboratory, Queensland, Australia and used as a normal control RNAs.

cDNA array hybridization

Total RNA from cell lines, melanocytes and fibroblasts was extracted using the Atlas Pure Total RNA Labeling System (Clontech) and DNase I (Roche) treated. The quality and integrity of the DNase treated RNA were checked by ethidium bromide agarose gel electrophoresis. Expression analysis was performed using the Atlas Human 1.2 (7850-1) and Atlas Human Cancer 1.2 (7851-1) nylon arrays (Clontech). Both filters contained 1176 genes (http://atlasinfo.clontech.com/atlasifo/array-list-action.do) of which 461 were present on both arrays. For each sample, 12.5 µg of total RNA was used in the cDNA probe synthesis with [α-^{32}P]dATP (NEN Life Science Products) and performed according to the Clontech Atlas cDNA Expression Arrays User Manual. Purification of the probe, hybridization and washes were done following the manufacturer's instructions (Clontech). Each cell line was simultaneously hybridized to both filters. After the washes, membranes were exposed for 1 to 3 nights to phosphoimager plates and scanned with a Storm Phosphoimaging System (Molecular Dynamics).

Analysis of cDNA arrays

The scanned gel images were converted to 16bit TIFF-file format. Signal intensities were quantified using the VisualGrid software version 2.1 (http://www.GPC-Biotech.com). The ArrayAn2 software (Boonefaes *et al*., manuscript in preparation) was used for further primary data analysis. In short, the spot intensities were corrected for the local background signal intensity, followed by a spot quality control step to exclude spots influenced by overshining effects of adjacent spots. Constitutive genes were selected (the 50% of spots showing the lowest coefficient of variation over all arrays) and used for normalization.

Expression data analysis

Genes with an expression value above background level in at least 9 of the analyzed samples were selected for further analysis. This resulted in a total of 1060 genes of which 410 were common genes. Cluster and Treeview software were used for unsupervised hierarchical clustering and visualization of the data[26]. Prior to clustering, genes were median centered and the expression data matrix was log transformed (base 2). Subsequently, complete linkage clustering using Pearson correlation coefficient as similarity metric was performed to the samples and genes. The complete expression data matrix is available as tab delimited file from the authors on request.

Unsupervised clustering of array data allows to find coherent patterns of gene expression but provides little information about the statistical significance. Therefore, we used the SAM algorithm (Significance Analysis of Microarrays, http://www-stat-class.stanford.edu/SAM/SAMServlet) which allows supervised identification of significantly expressed genes between predefined sample groups. In order to include less representative genes for the SAM analysis the filter threshold was lowered by including genes expressed above background in at least 4 of the analyzed samples (1513 genes).

Real-time quantitative RT-PCR

Sixteen SAM identified genes were selected and quantified by real-time quantitative RT-PCR on the same 21 RNA samples as for array hybridizations. Primer sequences for all 16 genes were designed with Primer Express 1.0 software (Applied Biosystems) using the default TaqMan parameters, with modified minimum amplicon length requirements (75 bp). The primer sequences are available from the authors on request and will be deposited in a public database for real-time PCR primers, which is under construction (www.realtimepcr.ht.st). Relative gene expression levels were determined using an optimized two-step SYBR green I RT-PCR assay as described by Vandesompele *et al*.[27]. The standard curve method (serial dilutions of a cDNA mixture containing 2 SCLC and 2 MCC samples) or the comparative Ct method was used for quantification. PCR reagents were obtained from Applied Biosystems as SYBR Green I mastermixes and used according to the manufacturer's instructions. PCR reactions were run on an ABI Prism 5700 Sequence Detection System (Applied Biosystems). To correct for differences in RNA quantities and cDNA synthesis efficiency relative gene expression levels were normalized using the geometric mean of 5 housekeeping genes (*UBC*, *HPRT1*, *GAPD*, *TBP* and *PBGD*) according to Vandesompele *et al*.[28].

Results

Validation of Atlas cDNA array expression data

Several points of evidence indicate good reliability of the array gene expression data:

1) Of the 1060 genes used for hierarchical clustering, 205 were present on both arrays (Human and Human Cancer 1.2 array, respectively). Hierarchical cluster analysis showed that 60 % of these common genes clustered next to each other (see Fig. 1D) indicating a minimal variation between the two filters after normalization. In order to assess more accurately the variation of two different filter experiments the Spearman correlation coefficient was calculated between the common genes on the 2 different filters for each sample. The mean correlation coefficient was 82.4 % which indicates a good correlation and demonstrates the reproducibility and the reliability of our results.
2) Sixteen SAM identified genes were randomly selected and quantified by real-time quantitative RT-PCR. The mean Spearman correlation coefficient between the array gene expression levels and real-time RT-PCR expression levels for these genes was 79.5 % indicating the reliability of the array gene expression (data not shown). Real-time RT-PCR analysis will be extended to additional cell lines and biopsies of MCC and SCLC in the near future in order to confirm the classification potential of these genes.
3) Two cell lines MCC14/1 and MCC14/2, derived from the same tumor, were found to cluster next to each other illustrating a highest degree of similarity as should be expected (see Fig. 1B).
4) Our gene expression analysis confirms the differential gene expression of *ASH1* in SCLC versus MCC already reported in the literature (see discussion).

Gene expression patterns of MCC, SCLC and NB cell lines

Hierarchical clustering was used to interpret the patterns of expression. Clustering of the 21 samples was based on the gene expression levels of 1060 pre-selected genes. Fig. 1A shows the complete cluster diagram. The dendogram (Fig. 1B) summarizes the degree of similarity in gene expression among the 21 analyzed samples. Three main groups were observed. Group 1 contained the two normal control samples (NFF and melanocytes) and four MCC cell lines (MCC13, MCC14/1, MCC14/2 and MCC26), indicating that these samples share a common gene expression pattern. We noticed that the MCC cell lines were all Type IV, adherent growing cell lines characterized by loss of expression of some neuroendocrine markers (i.e. Variant cell lines) (see Table 1). The observation of a number of overlapping gene expression patterns between the two normal samples and these four Variant, adherent growing MCC cell lines can possibly be explained by the fact that fibroblasts (NFF) and melanocytes also grow as attached monolayers and divide rapidly which is also the case for the Variant MCC cell lines. This in contrast with the Classic MCC cell lines included in this study, which all grow in suspension. The growth behavior of NFF, melanocytes and adherent growing MCC cell lines can partially result in a number of similar expression levels of genes typically involved in attachment such as integrins and adhesion molecules and of genes involved in cell division such as cyclins. This could explain the observed sample clustering in Group 1. Group 2 included two SCLC cell lines (GLC4 and NCI-H146), one MCC cell line (UISO) and all NB cell lines of which SK-N-SH formed a subcluster with UISO. Although UISO is a Type IV Variant MCC cell line some neuroendocrine dense-cored granules were observed[19], normally absent in Type IV cell lines. Expression of some neuroendocrine markers by these granules could possibly explain the clustering of UISO with other neuroendocrine tumors such as neuroblastoma.

Group 3 consisted of 5 MCC cell lines T95-45, MKL-2, MKL-1, MCC5 and MCC6 all classified previously as Classic MCC cell lines and the two SCLC cell lines CorL88 and NCI-H69.

The classification of MCC into two subgroups apparently coincided with the phenotypes, either Variant or Classic, of the cell lines. A subset of genes which is differentially expressed in Classic MCC cell lines as compared to all other samples is shown in Fig. 1C. Some of these genes are involved in neuronal development while others encode for ligand or voltage-gated ion channels. Similar, a selection of genes differentially expressed in the Variant MCC cell lines is shown in Fig. 1D. This cluster contains many oncogenes, transcription factors, cell cycle regulating kinases, and apoptosis-associated genes.

Identification of differentially expressed genes in SCLC *versus* MCC

From the above data we could conclude that gene expression differences between SCLC and MCC were not evidently by hierarchical cluster analysis. In order to identify a set of markers whose expression pattern could distinguish SCLC from MCC, we adopted a supervised strategy. Briefly, two sample groups namely SCLC (n=4) and MCC (n=10) were predefined. Subsequently, a two-class SAM-analysis on the log transformed data matrix containing 1513 genes (see Material and Methods) was performed. SAM identified genes were further selected as highly significant differentially expressed genes in SCLC versus MCC when a differential expression pattern was present in at least three of the four SCLCs as compared to MCC or in at least half of the MCC cell lines as compared to SCLC. Eight genes were higher expressed and 15 genes were lower expressed in SCLC as compared to MCC. Re-clustering of these genes confirmed the classification of the SCLC and MCC cell lines (Fig. 2A). In three of the four SCLC cell lines high-level expression of the neuronal differentiation marker *ASH1* (*achaete-scute homolog 1*), the basic helix-loop-helix transcription factor *ID2* (*inhibitor of DNA binding protein 2*) (Fig. 2B) and *GPRP* (*glutathione peroxidase-related protein*) was observed as compared to the MCC cell lines. The lower expression level of *ASH1* in cell line GLC4 corresponded with the fact that this was a Variant SCLC cell line while all others were Classic ones with neuroendocrine phenotype (see Table 1). Other highly expressed markers in all SCLCs included the transcription factors *AP4* and *EGR1, histone H4*, *insulin-like growth factor binding protein 2* and *fms-related tyrosine kinase* also known as *vascular endothelial growth factor receptor 1*. Genes lower expressed in all SCLCs or higher expressed in most MCC cell lines included several intracellular kinase network members such as *PKCA* (Fig. 2C), *MAPKK3*, *YSK1* and cell cycle regulating kinases such as *cyclin G-associated kinase* (*GAK*) and *aurora-related kinase 1* (*ARK1*).

Identification of differentially expressed genes in Variant *versus* Classic MCC cell lines

The same strategy as outlined above was applied to identify genes differentially expressed in Variant versus Classic MCC cell lines. Thirty-three genes with an increased expression level in the Variant MCC cell lines and 56 genes with an elevated expression level in the Classic ones were identified. Hierarchical cluster analysis of these 89 genes clearly confirmed the classification of the MCC cell lines in their respectively groups (Fig. 3). The genes with a higher expression in the Variant cell lines were particularly involved in cell cycle control and proliferation. Genes with kinase activities but also genes encoding for ligand and voltage-gated ion channels, neuromediators, GDP/GTP exchangers and

signal transduction receptors showed an increased expression level in the Classic MCC cell lines (Fig. 3).

Discussion

In an attempt to identify (1) new molecular markers whose gene expression patterns could improve differential diagnosis between MCC and SCLC and (2) typically gene expression signatures associated with Variant and Classic MCC cell lines we adopted a supervised data mining strategy.

This led to the identification of 23 genes that were shown to have a significantly altered gene expression between MCC and SCLC. Eight were found to be higher expressed in SCLC as compared to MCC. Our data confirmed the increased expression of the neuroendocrine differentiation marker *ASH1* in SCLC[29, 30] and absence of *ASH1* expression in MCC cell lines[12]. The *ID2* basic helix-loop-helix transcription factor was increased expressed in Classic SCLCs compared to MCCs. *ID2* plays a role in cell proliferation and differentiation and is able to disrupt the antiproliferative effects of retinoblastoma family members[31]. Disruption of the *RB1* pathway through increased expression of *ID2* could be an important mechanism in neuroendocrine SCLCs but not in MCC. The higher expression level of glutathione peroxidase (*GPRP*) in SCLC compared to MCC could reflect a mechanism of resistance of these SCLC cell lines to chemotherapeutic agents such as cisplatin. A similar observation was already described for another glutatione-related enzyme, glutathione S-transferase of which an increased expression in SCLC cell lines was seen after treatment with cisplatin[32, 33]. These findings warrants further investigation in SCLC and MCC cell lines and could suggest that alterations in glutathione metabolism do not play a role in MCCs in acquiring resistance to cisplatin. For the five remaining genes (*EGR1*, *FLT1*, *IGFBP2*, *histone H4* and *AP4*) higher expressed in all SCLC cell lines as compared to MCC no previous involvement in SCLC has been described. Early growth response 1 (*EGR1*) was frequently overexpressed in prostate carcinoma and played an important role in tumor initiation and progression. High level expression of *EGR1* in prostate carcinoma was the result of loss of a novel corepressor molecule *NAB2* that modulates *EGR1* activity[34]. A similar mechanism could be involved in SCLC tumorigenesis. *FLT1* or *vascular endothelial growth factor receptor 1* binds the vascular endothelial growth factor (*VEGF*), an important angiogenesis inducer. Frequent expression of *FLT1* was previously observed in squamous cell lung carcinoma, pointing at a possible autocrine role of *VEGF*[35]. Clinical trails with inhibitors against VEGF and VEGF receptors are ongoing in lung cancer[13]. The low expression of *FLT1* in MCC suggests the involvement of other angiogenesis inducers in MCC. The observed high level expression of *IGFBP2* in SCLC has also been reported in glioblastoma multiforme and might implicate involvement of the insulin growth factor (*IGF*) pathway in SCLC[36]. Further investigation of the transcription factor *AP4*, and *histone H4*, involved in cellular tracking and chromatin remodeling respectively[37, 38], should elucidate their role in SCLC or MCC biology.

Fifteen genes with increased expression in MCC were detected as compared to SCLC. None of these genes was previously shown to be implicated in SCLC and MCC biology. One striking finding was the differential expression of *PKCα* in MCC compared to SCLC cell lines. The protein kinase C is a key protein involved in regulation of cell growth and activation of the MAP kinase pathway[39], and the finding of increased expression is in keeping with the observed increased expression for *MAPKK3*. Both overexpression and downregulation of *PKCα* have been observed in tumor cells and

correlated with malignant transformation and proliferative activity of *PKCα*. *PKCα* could thus be involved in SCLC and MCC, albeit through a different mechanism in each of these tumor types. The *CDC25B* phosphatase gene activates cylin-dependent kinases at different points of the cell cycle. High expression of *CDC25B* has been reported in non-small cell lung carcinomas and was associated with unfavorable prognosis[40]. This could also be true for MCC in whom *CDC25B* is highly expressed compared to SCLC. The integrin linked kinase (*ILK*) is yet another interesting gene in relation to cancer biology. *ILK* is involved in adhesion of cells to the extracellular matrix and is integrated into the *PI3K* pathway as an activator of *AKT*. Recent studies showed that activation of *AKT* was one of the key elements during the different steps of mouse skin oncogenesis[41]. Enhanced expression of *ILK* in MCC followed by activation of *AKT* could thus play an important role in MCC development. Since the *PTEN* gene is rarely mutated in MCC[42], increased expression of *ILK* could therefore be a possible alternative mechanism for disruption of the *PI3K*/*AKT* kinase pathway in MCC. Further investigation of these genes and other SCLC and MCC classifier genes such as *RAC1*, *ITGA3*, *ARK*1, *p68 kinase*, *BDNF*, *CDH2*, *NRGN*, *GAK*, *GPRK6*, and *YSK1* should elucidate their role in SCLC and/or MCC biology. Finally, some of these genes or disrupted pathways could serve as potential targets for new rational therapeutic interventions in SCLC and/or MCC.

The present analysis revealed for the first time particular gene expression profiles for Variant and Classic MCC cell lines, respectively. We identified 89 highly significant differentially expressed genes of which 33 were higher expressed in the Variant MCC cell lines and 56 higher expressed in the Classic types. Upregulated genes in the Variant cell lines were involved in cell cycle control (*CCND1*, *CCNB1* and *G1 to S phase transition protein 1 homolog*) and cell proliferation (*HSP60*, *CSK2*, *MMP11*, *DBPA*, *MAPK9*, *FRA1* and *MAP kinase 38*). Some of these upregulated genes are possibly correlated with the shorter doubling time and aggressive nature of the Variant MCC cell lines as illustrated by the high cloning efficiency *in vitro* of these cell lines and poorly response to radiation[11]. In addition, we observed high expression of *vimentin*, a mesenchymal marker, together with *FRA1* (*FOS-related antigen 1*). A tight correlation of *vimentin* and *FRA1* expression was also recently found in highly invasive breast cancer cell lines pointing at a possible role in tumor progression and enhanced cell migration of these cancer cells[43]. These two genes could be significant prognostic markers for the more aggressive MCC Variant types. Increased expression of vimentin has also been previously observed by immunochemical studies in Variant SCLC cell lines[15, 44] and as a result of a suppression subtractive hybridization experiment comparing a Variant to a Classic SCLC cell line[16]. These observations could point at a similar mechanism of tumor progression or metastatic properties between MCC and SCLC Variant phenotypes. High level expression of *CCND1*, *CCNB1*, *the Axl receptor tyrosine kinase* and *MAP kinase p38*, genes involved in promoting cell growth and cell proliferation, was observed in Variant cell lines and could represent an important biological subset of markers for this aggressive group of MCCs.

A subset of genes with an increased expression level in the Classic cell lines are involved in signal transduction pathways leading to uncontrolled cell growth when overexpressed. This is exemplified by genes such as *MAPK3*, *MAPK7* and *MAPKK7* involved in the mitogen-activated protein (MAP) kinase pathway and genes such as *PI3-K p85 beta* and *PI4K-alpha* in the phosphatidylinositol 3-kinase (PIK3) pathway. In addition, Classic cell lines showed increased expression of genes encoding for neuromediators (*7B2*) and neurotransmitters (*NEC2*) and proteins involved in neuronal development such as lissencephalin and MARCKS-related protein (*MLP*). This reflects the neuroendocrine and differentiated character of the Classic types. Ligand- and voltage gated ion channels and receptors essential for neurotransmission were also upregulated. Some of these ion

channels are known to play a role during mechanical stimulation of normal Merkel cell receptors[45, 46]. Their specific function in MCC tumor cells has yet to be elucidated.

The differential expression levels of some of these markers reflect the different biological and clinical properties of Variant and Classic MCC phenotypes. Interestingly, SCLC tumors derived from morphological Variant cell lines are more aggressive and patients have a worse prognosis[47]. Comparable to SCLC, lack or overexpression of Variant MCC markers could indicate a subset of more aggressive MCCs for which more intensive treatment and closer follow-up are warranted. Future investigations of overexpressed markers or deregulated pathways involved in Variant and Classic MCC cell lines could lead to potential targets for development of new therapeutic strategies specific for each subgroup.

In conclusion, we demonstrated the potential power of gene expression profiling for improved differential diagnosis between Merkel cell and small cell lung carcinoma. In addition, we generated a gene expression-based classification of two biological and clinical distinct subgroups of MCC. This could result in a more selective therapeutic treatment and improvement of MCC patient outcome. Our study serves also as a first step to study differentially expressed genes involved in cell proliferation, signal transduction and neurotransmission in further detail, finally leading to more insight into the complex and heterogeneous biology of MCC.

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Table 1. Characteristics of the MCC and SCLC cell lines used for gene expression profiling

Cell line	Morphological Type	Colony shape	Colony aggregation	Classification*
MCC cell lines				
MCC5	I	3-d	Tight	Classic
MCC6	I	3-d	Tight	Classic
MCC13	IV	Flat	NA°	Variant
MCC14/1	IV	Flat	NA	Variant
MCC14/2	IV	Flat	NA	Variant
MCC26	IV	Flat	NA	Variant
UISO	IV	Flat	NA	Variant
MKL-1	III	2-d	Loose	Classic
MKL-2	III	2-d	Loose	Classic
T95-45	II	3-d	Loose	Classic
SCLC cell lines				
NCI-H69	II	3-d	Loose	Classic
NCI-H146	II	3-d	Loose	Classic
CorL88	III	2-d	Loose	Classic
GLC4	III	3-d	Loose	Variant

° Not applicable, adherent growing cell lines

*Classic MCC cell lines have elevated expression levels of neuroendocrine markers including neurone-specific enolase and Chromogranin A and contain neurosecretory granules. Variant MCC cell lines have a selective loss of neuroendocrine markers including Chromogranin A and do not contain neurosecretory granules as evidenced by electron microscopy (Leonard *et al*., 1997). Classic SCLC cell lines express elevated levels of L-dopa decarboxylase and bombesin while variant ones have undetecable levels of L-dopa decarboxylase and bombesin (Carney *et al*., 1985).

Legends to the figures

Figure 1.
Hierarchical clustering sorted 1060 selected genes and 10 Merkel cell carcinoma (MCC) cell lines, 4 small cell lung carcinoma (SCLC) cell lines, 5 neuroblastoma (NB) cell lines, melanocytes and neonatal foreskin fibroblasts (NFF) based on similarity in gene expression. Gene clusters relevant to Classic and Variant MCC subtypes were extracted from the large gene cluster of 1060 genes in the regions indicated by the colored bars and expanded on the right to include gene names. A row in the cluster indicates expression of a specific gene across all 21 samples. A column indicates the sample in which the gene is expressed. Red, green, and black squares indicate that expression of the gene is greater, less than, or equal to the median level of expression across all 21 samples, respectively. Gray represents missing data. (A) Scaled-down representation of the 1060-gene cluster diagram; colored bars to the right identify the location of the inserts displayed in C and D. (B) Dendogram representing similarities in the expression patterns between experimental samples. The colored horizontal lines reflect the different (sub)groups of tumor cell lines (dark blue, Variant MCC; light blue, Classic MCC; red, SCLC; green, NB). (C) Detail of gene cluster relevant to Classic MCC cell lines (light blue bar). (D) Detail of gene cluster relevant to Variant MCC cell lines (dark blue bar).

Figure 2.
Hierarchical cluster analysis was performed on 23 genes identified by SAM as MCC and SCLC classifiers. (A) Cluster diagram showing the classification of SCLC and MCC cell lines and their differentially expressed genes (red vertical bar, SCLC cell lines; blue vertical bars, MCC cell lines) (B) Histogram representing the normalized and log transformed (base 2) array gene expression level of *ID2* (*Inhibitor of DNA binding protein 2*) and (C) of *PKCA* (*Protein kinase C alpha polypeptide*) in MCC and SCLC cell lines.

Figure 3.
Hierarchical cluster analysis of 89 genes identified by SAM to classify the two distinct MCC subtypes. (light blue vertical bar, Classic MCC cell lines; dark blue vertical bar; Variant MCC cell lines).

Figure 1

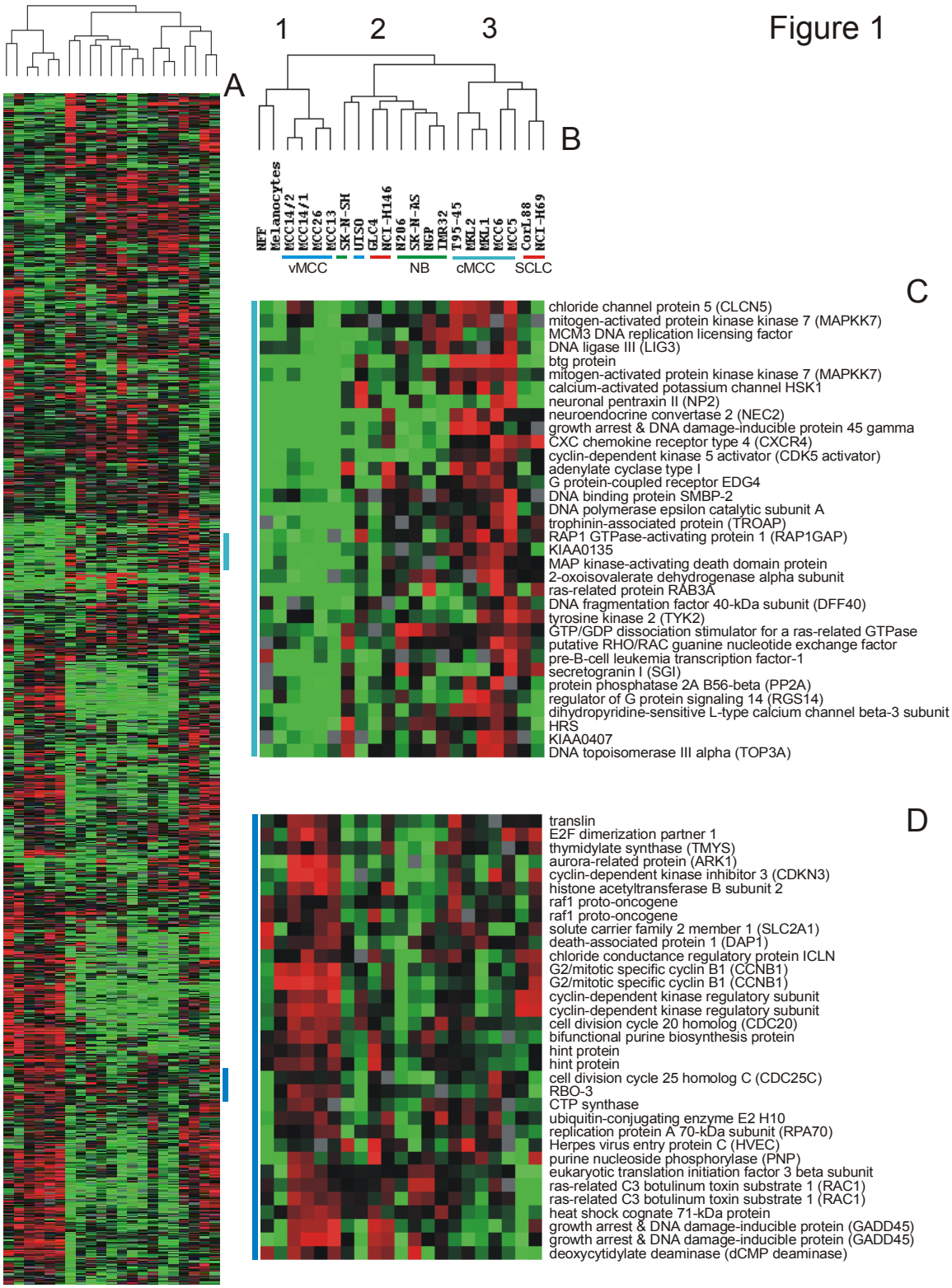
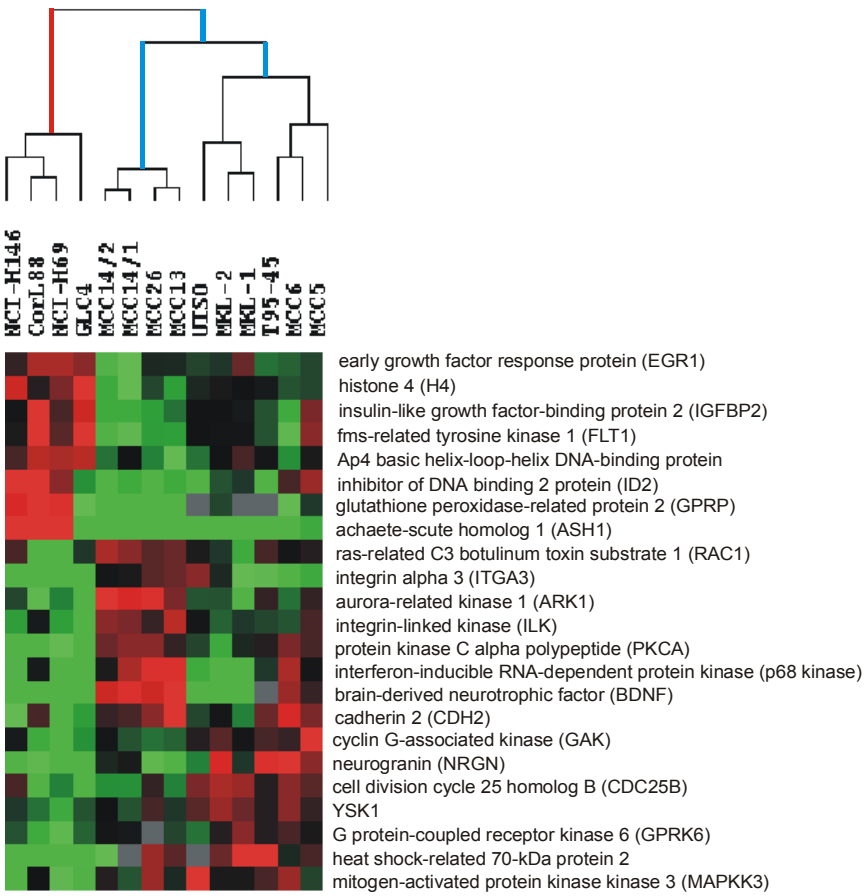
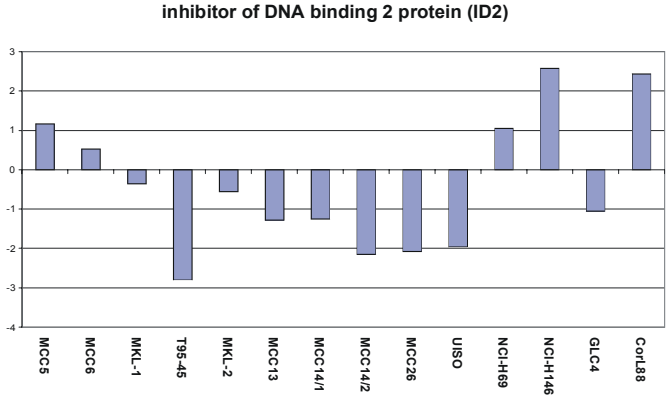

1
2
3
A
B
NFF
Melanocytes
MCC14/2
MCC14/1
MCC26
MCC13
SK-N-SH
UISO
GLC4
NCI-H146
N206
SK-N-AS
NGP
IMR32
T95-45
MKL2
MKL1
MCC6
MCC5
CorL88
NCI-H69
vMCC
NB
cMCC
SCLC
C
chloride channel protein 5 (CLCN5)
mitogen-activated protein kinase kinase 7 (MAPKK7)
MCM3 DNA replication licensing factor
DNA ligase III (LIG3)
btg protein
mitogen-activated protein kinase kinase 7 (MAPKK7)
calcium-activated potassium channel HSK1
neuronal pentraxin II (NP2)
neuroendocrine convertase 2 (NEC2)
growth arrest & DNA damage-inducible protein 45 gamma
CXC chemokine receptor type 4 (CXCR4)
cyclin-dependent kinase 5 activator (CDK5 activator)
adenylate cyclase type I
G protein-coupled receptor EDG4
DNA binding protein SMBP-2
DNA polymerase epsilon catalytic subunit A
trophinin-associated protein (TROAP)
RAP1 GTPase-activating protein 1 (RAP1GAP)
KIAA0135
MAP kinase-activating death domain protein
2-oxoisovalerate dehydrogenase alpha subunit
ras-related protein RAB3A
DNA fragmentation factor 40-kDa subunit (DFF40)
tyrosine kinase 2 (TYK2)
GTP/GDP dissociation stimulator for a ras-related GTPase
putative RHO/RAC guanine nucleotide exchange factor
pre-B-cell leukemia transcription factor-1
secretogranin I (SGI)
protein phosphatase 2A B56-beta (PP2A)
regulator of G protein signaling 14 (RGS14)
dihydropyridine-sensitive L-type calcium channel beta-3 subunit
HRS
KIAA0407
DNA topoisomerase III alpha (TOP3A)
D
translin
E2F dimerization partner 1
thymidylate synthase (TMYS)
aurora-related protein (ARK1)
cyclin-dependent kinase inhibitor 3 (CDKN3)
histone acetyltransferase B subunit 2
raf1 proto-oncogene
raf1 proto-oncogene
solute carrier family 2 member 1 (SLC2A1)
death-associated protein 1 (DAP1)
chloride conductance regulatory protein ICLN
G2/mitotic specific cyclin B1 (CCNB1)
G2/mitotic specific cyclin B1 (CCNB1)
cyclin-dependent kinase regulatory subunit
cyclin-dependent kinase regulatory subunit
cell division cycle 20 homolog (CDC20)
bifunctional purine biosynthesis protein
hint protein
hint protein
cell division cycle 25 homolog C (CDC25C)
RBO-3
CTP synthase
ubiquitin-conjugating enzyme E2 H10
replication protein A 70-kDa subunit (RPA70)
Herpes virus entry protein C (HVEC)
purine nucleoside phosphorylase (PNP)
eukaryotic translation initiation factor 3 beta subunit
ras-related C3 botulinum toxin substrate 1 (RAC1)
ras-related C3 botulinum toxin substrate 1 (RAC1)
heat shock cognate 71-kDa protein
growth arrest & DNA damage-inducible protein (GADD45)
growth arrest & DNA damage-inducible protein (GADD45)
deoxycytidylate deaminase (dCMP deaminase)

Figure 2

A


NCI-H146
CorL88
NCI-H69
GLC4
MCC14/2
MCC14/1
MCC26
MCC13
UISO
MKL-2
MKL-1
T95-45
MCC6
MCC5
early growth factor response protein (EGR1)
histone 4 (H4)
insulin-like growth factor-binding protein 2 (IGFBP2)
fms-related tyrosine kinase 1 (FLT1)
Ap4 basic helix-loop-helix DNA-binding protein
inhibitor of DNA binding 2 protein (ID2)
glutathione peroxidase-related protein 2 (GPRP)
achaete-scute homolog 1 (ASH1)
ras-related C3 botulinum toxin substrate 1 (RAC1)
integrin alpha 3 (ITGA3)
aurora-related kinase 1 (ARK1)
integrin-linked kinase (ILK)
protein kinase C alpha polypeptide (PKCA)
interferon-inducible RNA-dependent protein kinase (p68 kinase)
brain-derived neurotrophic factor (BDNF)
cadherin 2 (CDH2)
cyclin G-associated kinase (GAK)
neurogranin (NRGN)
cell division cycle 25 homolog B (CDC25B)
YSK1
G protein-coupled receptor kinase 6 (GPRK6)
heat shock-related 70-kDa protein 2
mitogen-activated protein kinase kinase 3 (MAPKK3)

B


inhibitor of DNA binding 2 protein (ID2)
3
2
1
0
-1
-2
-3
-4
MCC5
MCC6
MKL-1
T95-45
MKL-2
MCC13
MCC14/1
MCC14/2
MCC26
UISO
NCI-H69
NCI-H146
GLC4
CorL88

C

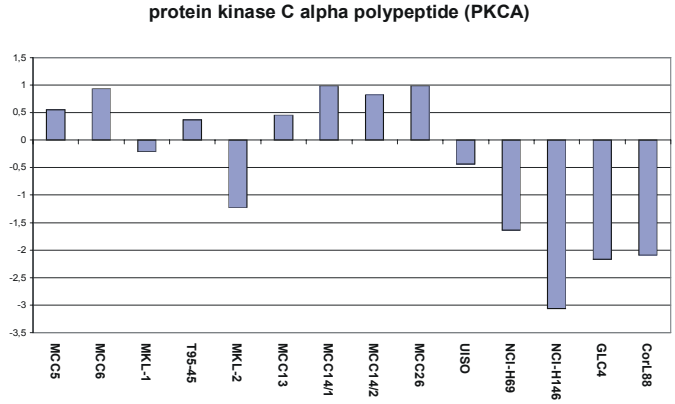
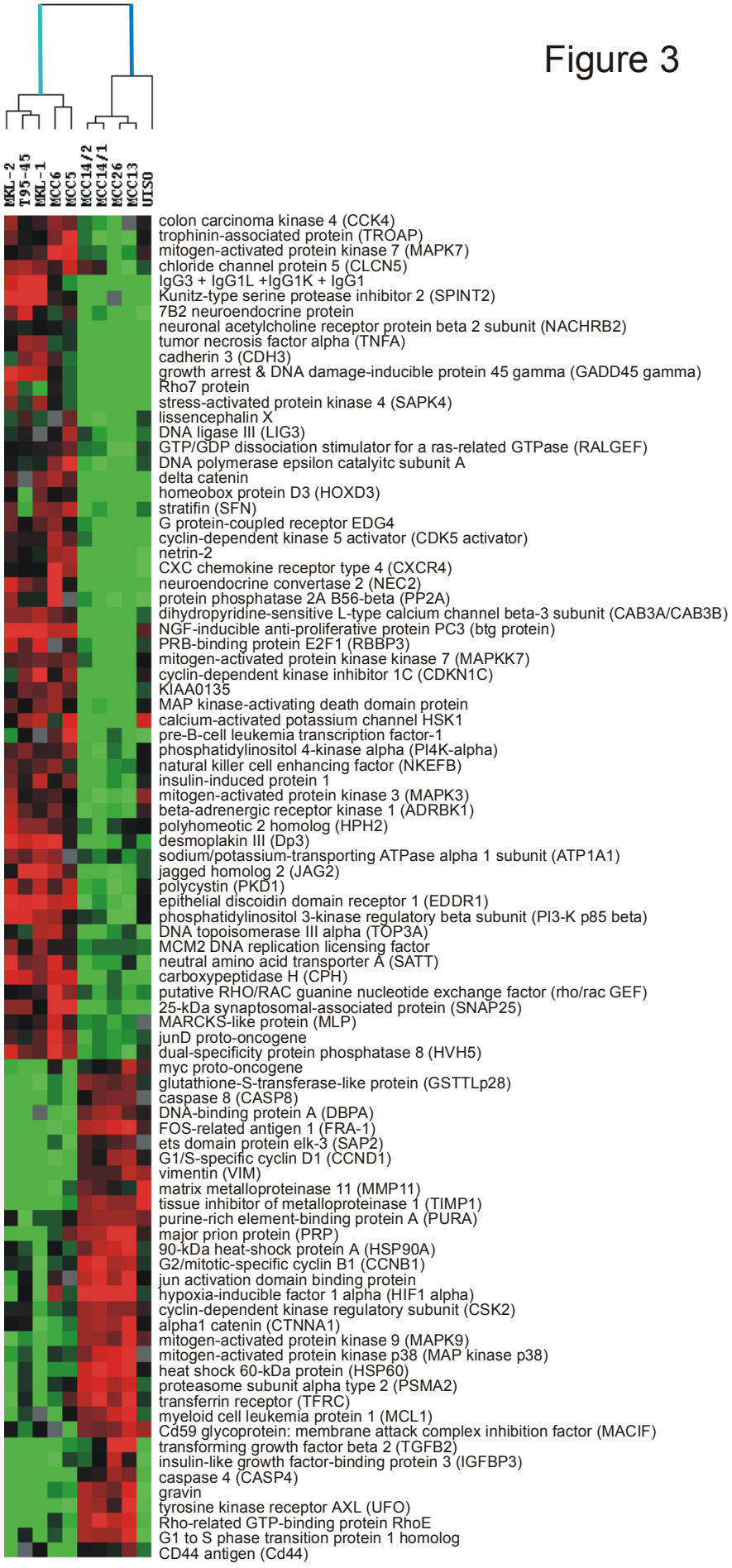

protein kinase C alpha polypeptide (PKCA)
1,5
1
0,5
0
-0,5
-1
-1,5
-2
-2,5
-3
-3,5
MCC5
MCC6
MKL-1
T95-45
MKL-2
MCC13
MCC14/1
MCC14/2
MCC26
UISO
NCI-H69
NCI-H146
GLC4
CorL88

Figure 3


MKL-2
T95-45
MKL-1
MCC6
MCC5
MCC14/2
MCC14/1
MCC26
MCC13
UISO
colon carcinoma kinase 4 (CCK4)
trophinin-associated protein (TROAP)
mitogen-activated protein kinase 7 (MAPK7)
chloride channel protein 5 (CLCN5)
IgG3 + IgG1L +IgG1K + IgG1
Kunitz-type serine protease inhibitor 2 (SPINT2)
7B2 neuroendocrine protein
neuronal acetylcholine receptor protein beta 2 subunit (NACHRB2)
tumor necrosis factor alpha (TNFA)
cadherin 3 (CDH3)
growth arrest & DNA damage-inducible protein 45 gamma (GADD45 gamma)
Rho7 protein
stress-activated protein kinase 4 (SAPK4)
lissencephalin X
DNA ligase III (LIG3)
GTP/GDP dissociation stimulator for a ras-related GTPase (RALGEF)
DNA polymerase epsilon catalyitc subunit A
delta catenin
homeobox protein D3 (HOXD3)
stratifin (SFN)
G protein-coupled receptor EDG4
cyclin-dependent kinase 5 activator (CDK5 activator)
netrin-2
CXC chemokine receptor type 4 (CXCR4)
neuroendocrine convertase 2 (NEC2)
protein phosphatase 2A B56-beta (PP2A)
dihydropyridine-sensitive L-type calcium channel beta-3 subunit (CAB3A/CAB3B)
NGF-inducible anti-proliferative protein PC3 (btg protein)
PRB-binding protein E2F1 (RBBP3)
mitogen-activated protein kinase kinase 7 (MAPKK7)
cyclin-dependent kinase inhibitor 1C (CDKN1C)
KIAA0135
MAP kinase-activating death domain protein
calcium-activated potassium channel HSK1
pre-B-cell leukemia transcription factor-1
phosphatidylinositol 4-kinase alpha (PI4K-alpha)
natural killer cell enhancing factor (NKEFB)
insulin-induced protein 1
mitogen-activated protein kinase 3 (MAPK3)
beta-adrenergic receptor kinase 1 (ADRBK1)
polyhomeotic 2 homolog (HPH2)
desmoplakin III (Dp3)
sodium/potassium-transporting ATPase alpha 1 subunit (ATP1A1)
jagged homolog 2 (JAG2)
polycystin (PKD1)
epithelial discoidin domain receptor 1 (EDDR1)
phosphatidylinositol 3-kinase regulatory beta subunit (PI3-K p85 beta)
DNA topoisomerase III alpha (TOP3A)
MCM2 DNA replication licensing factor
neutral amino acid transporter A (SATT)
carboxypeptidase H (CPH)
putative RHO/RAC guanine nucleotide exchange factor (rho/rac GEF)
25-kDa synaptosomal-associated protein (SNAP25)
MARCKS-like protein (MLP)
junD proto-oncogene
dual-specificity protein phosphatase 8 (HVH5)
myc proto-oncogene
glutathione-S-transferase-like protein (GSTTLp28)
caspase 8 (CASP8)
DNA-binding protein A (DBPA)
FOS-related antigen 1 (FRA-1)
ets domain protein elk-3 (SAP2)
G1/S-specific cyclin D1 (CCND1)
vimentin (VIM)
matrix metalloproteinase 11 (MMP11)
tissue inhibitor of metalloproteinase 1 (TIMP1)
purine-rich element-binding protein A (PURA)
major prion protein (PRP)
90-kDa heat-shock protein A (HSP90A)
G2/mitotic-specific cyclin B1 (CCNB1)
jun activation domain binding protein
hypoxia-inducible factor 1 alpha (HIF1 alpha)
cyclin-dependent kinase regulatory subunit (CSK2)
alpha1 catenin (CTNNA1)
mitogen-activated protein kinase 9 (MAPK9)
mitogen-activated protein kinase p38 (MAP kinase p38)
heat shock 60-kDa protein (HSP60)
proteasome subunit alpha type 2 (PSMA2)
transferrin receptor (TFRC)
myeloid cell leukemia protein 1 (MCL1)
Cd59 glycoprotein: membrane attack complex inhibition factor (MACIF)
transforming growth factor beta 2 (TGFB2)
insulin-like growth factor-binding protein 3 (IGFBP3)
caspase 4 (CASP4)
gravin
tyrosine kinase receptor AXL (UFO)
Rho-related GTP-binding protein RhoE
G1 to S phase transition protein 1 homolog
CD44 antigen (Cd44)

PART 5

General Discussion and Perspectives

5. General discussion and perspectives

As explained in the Introduction section, the data on genetic aberrations occurring in Merkel cell carcinoma (MCC) at the start of this investigation were sparse and few if any insights into the molecular pathology of this aggressive rare skin tumor were available at that time. In order to learn more about the underlying genetic basis and biology of MCC three major strategies were followed in this study.

1) Whole genome screening methods i.e. karyotyping, multiplex FISH (M-FISH) and comparative genomic hybridization (CGH), were used in order to identify recurrent patterns of chromosomal changes and specific involvement of particular genomic regions.
2) Functional candidate genes located in some of these putative critical regions were investigated in order to determine their possible role in MCC.
3) Gene expression profiles of 1891 unique genes known to be involved in cell cycle control, cell-cell interactions, apoptosis, and signal transduction pathways were analyzed in MCC and SCLC in order to identify genes and pathways involved in MCC and to search for molecular markers which could improve differential diagnosis between MCC and SCLC.

5.1 Chromosome region 1p

5.1.1 Mapping of critical regions on 1p

Although several MCCs were described with 1p deletions, including an MCC tumor with a deletion of the distal part of 1p (1p36.1→pter) as the sole chromosomal abnormality (Gibas *et al.*, 1994), the size and position of these deletions was only determined by G-banding. The only exception was the study of three other MCC tumors which were examined for loss of 1p by Southern blot analysis with six polymorphic probes (Harnett *et al.*, 1991). All cases were found to have LOH for at least one locus but due to insufficient density of the polymorphic probes no common region of deletion could be defined. In contrast to MCC, other more common tumors (e.g. neuroblastoma, melanoma, breast carcinoma, colon carcinoma and hepatocellular carcinoma) with 1p loss were extensively studied by FISH and high-polymorphic LOH markers. This led to the delineation of shortest regions of overlap (SRO) for the recurrent deletions on 1p in each tumor type and

allowed the determination of overlapping SROs for the different tumor types (Schwab *et al.*, 1996). These SROs provided critical evidence for the localization of putative tumor suppressor genes which could have been targeted by these common deletions.

In this study, we investigated two particular chromosome 1p36 rearrangements in an MCC tumor and cell line, respectively. In the tumor UHG-VM, karyotyped at the Center for Medical Genetics, an unbalanced translocation between two chromosomes 1 was observed with evidence for loss of the most distal 1p36 region only. This further supported the finding of Gibas *et al*. (1994) that this region was specifically implicated in MCC. Moreover, the fact that this chromosome 1 rearrangement was the only structural change in the karyotype of patient UHG-VM further stressed the importance of distal 1p loss in the development of MCC. Detailed FISH analysis with region specific probes and high-polymorphic microsatellite markers allowed the assignment of a deletion breakpoint distal to the enolase 1 (*ENO1*) gene located within chromosome subband 1p36.31 (see Paper 2.1.1), thus indicating that the 1p36.1→pter segment was lost in this tumor. This finding allowed us to delineate a minimal region of loss in MCC which could harbour a putative tumor suppressor locus. Recently, a new MCC patient was investigated in our laboratory. The karyotype of the MCC tumor of this patient showed an apparently identical der(1)t(1;1) (p36;q21) resulting in loss of distal 1p and gain of the entire chromosome 1 long arm. This observation provides further support for the involvement of loss of distal 1p36 in MCC. Refined mapping of the breakpoint is ongoing. Interestingly, this type of chromosomal rearrangements involving band regions 1p36, 1p11-12 and 1q21 were also frequently observed in melanoma (Zhang *et al.*, 1999). These regions contain homologous sequences associated with chromosomal instability. A mechanism of preferentially homologous recombination between these regions could directly play a role in the generation of these types of chromosomal rearrangements and could also be implicated in MCC.

The MCC cell line, UISO, was initially believed to carry an unbalanced 1;17 translocation (Ronan *et al.*, 1993). FISH analysis, however, showed that the abnormal chromosome 1 resulted from insertion of repetitive DNA sequences into the 1p36 region (see Paper 2.1.3). Additional FISH analysis mapped the breakpoint to 1p36.2 between markers *RIZ* and *A12M2* within a cluster of repetitive genes and pseudogenes (van der Drift *et al.*, 1994). Interestingly, this insertion breakpoint coincides with the 1p36 breakpoint of the constitutional 1;17 translocation previously observed in a patient with neuroblastoma in

our laboratory (Laureys *et al.*, 1995; van der Drift *et al.*, 1995; Van Roy *et al.*, in press). Cloning of the 1;17 translocation breakpoints in this neuroblastoma patient revealed that the 1p36.2 breakpoint disrupted a gene belonging to a new gene family. Further characterization of this gene and studies to unravel its possible role in tumor development are currently ongoing. Future research should resolve whether the same gene is affected by both rearrangements and whether this gene plays a role in the development of both tumor types and possibly also in other tumors showing 1p loss. Consequently, the molecular analysis of these two MCC cases already indicated that two distinct regions on 1p36, each harbouring a putative tumor suppressor locus, could be involved in MCC. This picture was further complicated by a second more extensive LOH study performed with 1p markers, in which our group contributed. This study revealed a complex pattern of LOH with several regions of loss occurring within a given tumor and the observation of a total of three SROs (Leonard *et al.*, 2000). The frequent finding of loss of distal 1p36 in MCC led us to assume that a putative tumor suppressor gene located in this particular region could play a role in MCC development. Interestingly, this distal region was also frequently deleted in neuroblastoma and was thought to harbour an imprinted tumor suppressor gene (Caron *et al.*, 1995). A recently identified (imprinted) candidate tumor suppressor gene for this region was *TP73* (Kaghad *et al.*, 1997) and is discussed below. In addition to the *TP73* gene, mutation analysis and expression analysis by real-time quantitative PCR was performed for the *RIZ1* (retinoblastoma protein-interacting zinc finger) (1p36.2) candidate tumor suppressor gene. No mutations were found in MCC, but one mutation was detected in a neuroblastoma cell line. This finding together with the observation of other *RIZ1* mutations in ten diffuse large B-cell lymphomas, one osteosarcoma cell line and the development of a broad range of tumors in *RIZ1*$^{-/-}$ mice provided evidence for a tumor suppressor function of *RIZ1* in human cancers (Greenwood, 2001; Steele-Perkins *et al.*, 2001).

5.1.2 Candidate gene analysis: *TP73*

In view of the structural and functional homology of *TP73* with *TP53*, the location of *TP73* in SROs for 1p deletions occurring in various cancers and the initially described monoallelic expression of the gene, *TP73* was regarded as a strong candidate tumor suppressor gene. In order to test this hypothesis, mutation analysis studies for *TP73* were performed. Thus far, mutations were found in the carboxy-terminal region of *TP73* in two neuroblastoma tumors, one breast carcinoma and three lung cancer cell lines (Han *et al.*, 1999; Ichimiya *et al.*, 1999; Yoshikawa *et al.*, 1999). Functional analysis showed that at least one of these mutations (P425L) reduced the transactivation and growth-suppressive activity of *TP73* in *TP53*-deficient SAOS-2 cells (Naka *et al.*, 2001). As *TP73* also mapped within the small distal 1p36 deletions observed in MCC, we decided to screen the gene for inactivating mutations in five MCC cell lines and ten MCC tumors. In view of the possible complementary functions of *TP73* and *TP53*, the mutation status of *TP53* was also examined (see Paper 3.1.1). Mutation analysis of *TP73* revealed a *TP73* missense mutation located in the NH_2-terminal transactivation domain of the gene in one MCC tumor. The transcriptional transactivation function of the amino-terminal regions of *TP73* and *TP53* were shown to have an equally strong activity (Takada *et al.*, 1999). We hypothesized that the observed *TP73* missense mutation in MCC could lead to a reduction or complete loss of the transactivation function of the amino-terminal region in *TP73* which could lead to the disruption of certain specific growth-inhibitory functions of *TP73*. The MCC tumor mutated for *TP73* still expressed the remaining wild-type allele. Therefore analogous to *TP53*, this missense mutation could exert a dominant negative effect by an increased stability of the mutant protein compared to that of the wild-type protein. The MCC tumor harbouring the mutated *TP73* contained also a *TP53* non-sense mutation. Mutation of both genes was also observed in a patient with breast cancer and two lung cancer cell lines (Han *et al.*, 1999; Yoshikawa *et al.*, 1999). These findings suggest that *TP73* and *TP53* are not involved in the same tumor suppressor pathway or that *TP73* plays only a minor role in tumorigenesis. Four other *TP53* mutations were also identified in two MCC cell lines. Our results showed for the first time the occurrence of inactivating *TP53* mutations in MCC, suggesting that *TP53* may play a role in the pathogenesis or progression of a subset of MCCs. The finding of typical UVB induced mutations in one MCC cell line provided further evidence for sun-exposure in the etiology of this skin tumor. Recently, additional

UVB type mutations in the *TP53* gene have been identified in two other MCC cell lines by Popp *et al*. (2002). This further supports a role of UVB radiation in MCC development.

The fortuitous discovery of a first *TP53* family member, *TP73*, and its location in a region frequently deleted in many tumor types triggered a lot of attention in the cancer field. However, the present data are not convincing that loss of expression of *TP73*, like *TP53*, plays a crucial role in cancer development. Mutations are infrequently found in tumors with 1p loss and a recent report demonstrated that *TP73* deficient mice showed no increased tendency to develop spontaneous tumors, but exhibited neurological and inflammatory defects indicating involvement of *TP73* at specific stages of development (Yang *et al.*, 2000). Initially, *TP73* was assumed to be monoallelic expressed. Several studies, including ours, showed however biallelic expression of *TP73* (reviewed in Levrero *et al.*, 2000). Consequently, deletion of one allele, at least in these investigated cell types, would not be sufficient for complete silencing of the *TP73* locus. The recent discovery that certain splice variants of *TP73* may exert a dominant negative effect on *TP53* activity points at another mechanism through which *TP73* might be involved in tumorigenesis. (reviewed in Yang *et al*., 2002). In addition, elevated expression of TP73 was observed in various tumor tissues compared to the tissue of origin indicating that altered expression of TP73 rather than loss of function is involved in instability and tumorigenesis (Tannapfel *et al.*, 1999).

Our data revealed that the *TP73* gene itself is infrequently mutated in MCC. However, the observation of loss of distal 1p material, often reported as sole structural change in MCC, and in many other cancers suggests that this region is important. At the moment there are no reports of TP73 protein levels, or of different splice variants in MCC. If such studies would show no involvement of TP73 in MCC then we must assume that other, as yet unidentified tumor suppressor gene(s) are involved in MCC carcinogenesis.

5.2 Search for other chromosomal regions involved in MCC

5.2.1 Comparative genomic hybridization analysis

CGH analysis of 26 tumor samples and eight MCC cell lines led to the identification of a characteristic pattern of recurrent gains and losses in MCC (see Paper 2.1.3).
The following new information resulted from these CGH patterns:

(1) gene amplification was, in contrast to CGH findings in many other tumors, a rare event in MCC. This suggests that high level amplification of proto-oncogenes is not a major step in the oncogenesis of MCC;
(2) significant gains were detected for chromosomes 1 (63%), 19 (63%) and X (41%) and chromosomal regions 3q (33%), 5p (38%) and 8q (38%) which may lead to a dosage effect for genes located in these regions;
(3) frequent losses were observed for 3p (46%), 5q (21%), 8p (21%), 10 (33%), 11q (17%), 13q (33%) and 17p (25%) suggesting the presence and involvement of tumor suppressor genes;
(4) the pattern of gains and losses closely resembled those described in SCLC (Ried *et al.*, 1994; Levin *et al.*, 1995; Petersen *et al.*, 1997). This suggests that a number of common genetic changes or genes may play an important role in the tumorigenesis of both tumors.

Recurrent regions of loss and analysis of candidate tumor suppressor genes located in these regions are discussed in more detail below. The most frequently observed loss in MCC was loss of chromosomal region 3p, which is in agreement with previously reported LOH data by Leonard *et al*. (1996). A consensus deletion region at 3p11~14 was defined by CGH thus overlapping the 3p SRO defined in SCLC (Petersen *et al.*, 1997). The *FHIT* gene is located within this region and showed exonic loss in 80% of SCLCs (Sozzi *et al.*, 1996b) and 57% of MCCs (Sozzi *et al.*, 1996a). This suggests an important role for this gene in lung and Merkel cell carcinogenesis. A second more distal region of loss (3p21→pter) described in SCLC could not be attributed in MCC. However, as loss of the entire chromosome 3 arm was present in 38% of the MCCs possible involvement of tumor suppressor genes in this more distal region can not be excluded. In a recent study no mutations nor promotor hypermethylation of the von Hippel-Lindau (3p25-26) tumor

suppressor gene were detected in MCC (Hoebeeck *et al*., unpublished results). Inactivation by allele loss and promoter region methylation of *RASSF1A* (Dammann *et al.*, 2000; Burbee *et al.*, 2001) and *SEMA3B* (Tomizawa *et al.*, 2001), both located at 3p21, has been demonstrated in SCLC and will be investigated in future studies in MCC.

Chromosomal region 13q was the second most frequently lost region in MCC. The shortest region of overlap was 13q14~21, a region containing the *RB1* locus in agreement with LOH data reported by Leonard and Hayward (1997). These authors also showed by Western blot analysis absence of RB1 expression in all tested MCC cell lines. Inactivation of RB1 was also observed in 90% of SCLCs, resulting in deregulation of the *RB1*/*p16*^{INK4a}/c*yclinD1* cell cycle control pathway (see Figure 1-2, Part 1) (Fong *et al.*, 1999). Co-inactivation of RB1 and p16^{INK4a} is rarely observed in both SCLC and MCC but, unlike SCLC, p14^{ARF} expression was not lost in MCC (Shapiro *et al.*, 1995; Gazzeri *et al.*, 1998; Cook *et al.*, 2001). In addition, LOH analysis of the 9p region implicated the existence of tumor suppressor genes mapping both proximal and distal to the *CDKN2A* locus in MCC (Cook *et al.*, 2001).

Loss of chromosomal region 17p, harbouring *TP53*, was observed in one quarter of the examined cases. Mutation analysis of the *TP53* gene was performed by us in this study (see Paper 3.1.1) and revealed mutations in 19% of the examined MCC cases. These results suggested that *TP53* may play a role in the pathogenesis of a subset of MCCs.

A new, previously unexplored region of frequent chromosomal loss detected in MCC involved chromosome 10. Deletions of the long arm of chromosome 10 have been observed in many tumor types including SCLC, melanoma and prostate carcinoma. The recently discovered tumor suppressor gene *PTEN*, located at 10q23, was shown to be mutated in various tumors (reviewed in Ali *et al*., 1999). We proposed that this gene could also be affected in MCC and investigated therefore its possible role in MCC.

5.2.2 Candidate gene analysis: *PTEN*

LOH analysis of the 10q23 region was performed in 23 MCC patients. These tumors and eight additional cell lines were subsequently screened for *PTEN* mutations and intragenic homozygous deletions. A high frequency of LOH at the *PTEN* locus (43% of informative samples) was detected. Despite this high incidence of LOH, we found only one *PTEN* mutation located at exon 5 and one homozygous deletion for exon 9 in two different MCC tumors. The second *PTEN* allele was deleted in the patient harbouring the mutated *PTEN*

gene. The detected *PTEN* mutation was located in the phosphatase domain, 15 codons downstream of the protein tyrosine phosphatase (PTPase) core motif of *PTEN* and resulted in a stopcodon. This non-sense mutation could lead to a truncated protein missing a part of exon 5 and the following exons, resulting in complete loss of PTEN protein activity or a truncated protein with reduced phosphatase activity leading to a decrease or inactivation of the tumor suppressor function of *PTEN*. Alternatively, loss of the C-terminal region harbouring the C2 domain and PDZ motifs of PTEN could lead to PTEN instability and loss of phosphatase activity (Georgescu *et al.*, 1999).

Our results indicate that inactivation of *PTEN* by loss of one allele and by mutation or homozygous deletion of the remaining allele is a relatively rare event in MCC. However, we can not exclude that deregulation or inactivation of *PTEN* in MCC may involve other mechanisms such as a) mutations located in the promotor or intronic regions of *PTEN*; b) *PTEN* promotor methylation as recently reported in metastatic endometrial carcinomas (Salvesen *et al.*, 2001) or c) bi-allelic inactivation of *PTEN* through other epigenetic mechanisms as suggested in malignant melanomas without *PTEN* mutations or deletions (Zhou *et al.*, 2000). Presently, the above mentioned mechanisms have not been confirmed by many other research groups. Interestingly, haploinsufficiency of the *PTEN* gene as recently demonstrated for prostate cancer and other tumor types (Kwabi-Addo *et al.*, 2001) can also lead to tumor formation or progression. The latter possibility could explain the higher rates of LOH at 10q23.3 and the much lower rates of inactivation of the retained *PTEN* allele by mutations or homozygous deletions in a subset of MCCs with loss of chromosome 10. The relatively low expression levels for *PTEN* measured by cDNA expression arrays and real-time quantitative RT-PCR could be in keeping with the observed inactivation of one or both *PTEN* alleles in MCC. However, in the absence of data concerning *PTEN* expression levels in normal Merkel cells (both at the expression and protein level) no definitive conclusion can be drawn from the above expression results. Further studies are needed, including assessment of PTEN protein levels, to clarify the role of *PTEN* in MCC. Alternatively, one or more tumor suppressor genes located either proximal or distal to *PTEN* might be involved in the etiology of MCC since CGH and M-FISH (see Paper 2.1.3) revealed that loss of an entire copy of chromosome 10 was a frequent finding in MCC. The recent observation of a new MCC patient with a 1p loss and monosomy 10 as the only numerical change supports the fact that loss of chromosome 10 is an important event in MCC (Van Gele *et al.*, unpublished results). The observation of two MCC cases with unbalanced translocations resulting in loss of the 10q23→qter region

(see Paper 2.1.3) indicated that a tumor suppressor gene located distal to *PTEN* could be more likely involved in MCC. Putative candidate tumor suppressor genes located distal to *PTEN* include *DMBT1* (10q25.3-26.1) (Mollenhauer *et al.*, 1997), *MXI1* (10q24-25) (Eagle *et al.*, 1995), *LGI1* (10q24) (Chernova *et al.*, 1998) and *h-neu* (10q25.1) (Nakamura *et al.*, 1998) and could be targets for future investigations.

5.2.3 Multiplex FISH (M-FISH) analysis

To obtain a more detailed picture of the genetic changes that lead to MCC development, we combined karyotyping, CGH, M-FISH and FISH with region specific probes and analyzed six MCC cell lines and one MCC tumor (see Paper 2.1.3). The patterns of chromosomal changes in MCC were heterogeneous: simple karyotypic changes, multi-way translocations and karyotypes with various numerical and structural changes. The major chromosomal changes observed in this study were: chromosomal rearrangements involving the short arm of chromosome 1, leading in one case to loss of 1p36.3 material and the long arm of chromosome 3, gain of 5p material and loss of chromosome 10. The occurrence of isochromosomes of the short arm of chromosome 5 and gain of 3q23-26 material could correlate to a low level copy number gain of genes which might induce a growth advantage important for tumor initiation or progression. No high level gene amplifications however were detected. All these observations were in agreement with previously reported cytogenetic studies and/or CGH results described in Paper 2.1.2. In addition, M-FISH combined with karyotyping and CGH provided a detailed and accurate description of previously unidentified (complex) rearrangements and contributed to the limited cytogenetic data of MCC. This study should invite other investigators to characterize chromosomal rearrangements occurring in newly established MCC cell lines and tumors in a similar way if possible which would contribute to a better insight into the chromosomal events and mechanisms affecting and regulating MCC.

5.3 Differential gene expression analysis

Atlas cDNA filter expression arrays were successfully applied to determine the gene expression profiles of MCC and SCLC cell lines. Data mining analysis tools were used to identify significant differentially expressed genes between MCC and SCLC and to find typical gene expression signatures associated with the phenotypically different subgroups (i.e. Classic versus Variant) of MCC.

We identified a set of 23 powerful predictor genes, which could improve differential diagnosis between MCC and SCLC. For the majority of these genes no previous involvement was shown in MCC and SCLC biology although they have well known roles in angiogenesis and tumor progression. These genes are integrated into the insulin growth factor, MAP kinase or PI3K kinase pathways. Further investigation of these 23 classifier genes and pathways may provide insight into the altered cellular functions which lead to MCC or SCLC tumorigenesis. Some molecular markers or disrupted pathways could be potential targets for new therapeutic treatments and improve patient survival in MCC and/or SCLC. Of particular interest was the finding of an increased expression level of the integrin linked kinase (*ILK*) in MCC as compared to SCLC. *ILK* is integrated into the *PI3K* pathway as an activator of *AKT*. Enhanced expression of *ILK* in MCC followed by activation of *AKT* could thus play an important role in MCC development. Since our molecular analysis study of the *PTEN* gene showed that *PTEN* itself is infrequently mutated in MCC, increased expression of *ILK* could therefore be a possible alternative mechanism for disruption of the *PI3K/AKT* kinase pathway in MCC. cDNA expression profiling provided thus additional useful information.

Typical gene expression profiles were found to be associated with the Variant and Classic MCC cell lines, respectively. We identified a set of 89 highly significant differentially expressed genes, which allowed classification of the MCC cell lines into Variant and Classic subgroups. Genes mainly involved in cell cycle progression and cell proliferation showed higher expression levels in Variant MCC cell lines, mainly reflecting their more aggressive behavior. Genes involved in signal transduction, neurotransmission and neuronal development showed a higher expression level in Classic MCC types associated with their neuroendocrine and differentiated character. We assume that the differential

expression levels of some of these genes reflect, analogous to SCLC, the different biological and clinical properties of Variant and Classic MCC phenotypes. This would implicate a more aggressive treatment for tumors with a Variant morphology and a closer follow-up for this patient group. Further investigation on clinical samples should reveal which genes could serve as useful prognostic markers and potential targets for the development of new therapeutic interventions specific for each MCC subgroup. A further detailed molecular study of differential genes and pathways involved in Variant and Classic subtypes respectively, could contribute to a better insight into the molecular basis of their different malignant behavior.

Furthermore, a targeted search to identify similar upregulated genes or disrupted pathways involved in Variant and Classic MCC and SCLC cell lines would also be of interest to develop novel therapeutic interventions applicable for both tumors. The usefulness of such an approach was recently exemplified by studies of the tyrosine kinase inhibitor STI-571. This drug was originally developed as a tyrosine kinase inhibitor against the Abl tyrosine kinase activity and shown to be highly effective in treating patients with CML (Druker *et al.*, 1996). Interestingly, STI-571 also inhibited the tyrosine kinase activities of the PDGF and c-kit receptor. This led to the investigation of STI-571 in selected SCLC cell lines known to express the c-kit receptor. Experimental studies showed that STI-571 inhibited cell growth of these SCLC cell lines through a mechanism that involved inactivation of the tyrosine kinase c-kit and may be useful in a clinical trial for patients with SCLC (Wang *et al.*, 2000). The therapeutic effects of this new drug on other solid tumors expressing activated or mutated c-kit are currently ongoing. Parallel to this example, identification of common activated genes, receptors or pathways in SCLC and MCC could thus result in development of a common therapeutic drug benefiting both patient groups.

To conclude, a major finding of the research conducted in this thesis was the fact that MCC and SCLC shared many similarities both on the genomic and expression level. Both tumors are neuroendocrine tumors expressing a number of common immunohistochemical markers and belong to a family of "small round blue cell" tumors. The overlapping CGH results of MCC and SCLC, the involvement of common disrupted pathways and the identical heterogeneity of MCC and SCLC cell lines suggest that both tumor entities are genetically related. This proposed model could indicate that MCC and SCLC are possibly derived from a similar precursor cell. Interestingly, a number of common typical genetic changes between MCC and SCLC including loss of 3p, 10q, 17p and gain of 3q, 5p and 8q, are also often found in other tumor types. The significance of these observations remains to be determined.

5.4 Perspectives

Refined delineation of critical regions

Our molecular cytogenetic analysis contributed to the identification of new chromosomal candidate regions involved in MCC. This analysis will in the future be extended by the use of genomic arrays (array CGH). This approach could lead to a further refined delineation of known critical regions, the identification of new candidate regions or even homozygous deletions. Putative candidate genes located in new identified regions will be targets for future investigations.

Candidate gene analysis

Known chromosomal regions involved in MCC will be further analyzed and candidate genes residing in these regions will be examined.

Specifically, the 10q region distal to *PTEN* will be subjected to further LOH analysis. Depending on these results, candidate tumor suppressor gene such as *DMBT1* (10q25.3-26.1), *MXI1* (10q24-25), *LGI1* (10q24) and *h-neu* (10q25.1) could be further examined by mutation analysis and/or methylation-specific PCR.

Loss of chromosomal region 3p is a recurrent finding in MCC. Therefore, the possible role of candidate tumor suppressor genes such as *RASSF1A*, *SEMA3B* and *BLU*, all located at 3p21.3 (Lerman and Minna, 2000), will be examined in MCC by mutation analysis, methylation-specific PCR and homozygous deletion screening.

Validation of differentially expressed genes in MCC and SCLC by real-time quantitative RT-PCR

Presently, real-time RT-PCR is ongoing in our laboratory to quantify the expression levels of differentially expressed genes in the analyzed cell lines. This analysis will be extended to additional tumor cell lines and tumor biopsies of SCLC and MCC in order to assess the diagnostic value of these biological markers in clinical samples. Ultimately, a diagnostic custom cDNA microarray will be developed at our laboratory leading to an improved diagnosis of MCC and SCLC and patient outcome prediction.

Evaluation of clinicopathological significance of biological markers in MCC and SCLC

In addition, biological markers for which antibodies are available will be further investigated on the protein level by Western blots or immunohistochemistry. In collaboration with the Department of Pathology (Ghent) paraffin-embedded tumor material of MCC, SCLC and other neuroendocrine tumors will be collected. These archival tissue specimens will be used to develop a tissue microarray (TMA). TMA technology allows a rapid visualization of molecular markers in hundreds to thousands of tumor specimens at a time (Nocito *et al.*, 2001; Torhorst *et al.*, 2001). Immunohistochemical analysis of our newly identified biological markers will be performed on the TMA slides in order to study their clinicopathological significance. In addition, TMA slides can also be used for fluorescence in situ hybridization and RNA in situ hybrididzation.

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SUMMARY

Merkel cell carcinoma (MCC) is a rare aggressive neuroendocrine skin tumor mostly affecting elderly people. The disease occurs predominantly on the sun-exposed areas of the skin, suggesting UV exposure in its etiology. MCC is thought to be derived from Merkel cells which act as mechanoreceptors in the skin. At the start of this doctoral study, the molecular events leading to MCC were poorly understood as only limited cytogenetic studies were available. These studies revealed recurrent involvement of 1p abnormalities, often leading to loss of chromosomal region 1p36, in MCC but little or no information on involvement of other chromosomal regions could be obtained from these data. One of the initial aims of this study was to gain new information concerning genetic aberrations occurring in MCC. Therefore, various (cyto)genetic and molecular techniques were used to study MCC. In addition, it was reported that MCC shared clinical and immunohistochemical features with small cell lung carcinoma (SCLC), another neuroendocrine tumor and both tumors were often difficult to differentially diagnose. To understand more about the molecular biology of both tumors, gene expression profiles of MCC and SCLC were determined and further analyzed in detail.

In view of the evidence for recurrent involvement of 1p in MCC this region was first targeted in our study. This resulted in the finding that two distinct regions at 1p36 were implicated. In one MCC tumor, loss of the most distal part of 1p36 (1p36.31→pter) was observed as the result of an unbalanced structural rearrangement. This region was previously shown to be deleted in many other tumor types and was thought to harbour an (imprinted) tumor suppressor gene. The identification of a 1p36.2 insertion breakpoint in the MCC cell line UISO indicated a second, more proximal region involved in MCC. This breakpoint was mapped within a cluster of repetitive genes and coincided with the 1p breakpoint of a constitutional 1;17 translocation found in a patient with neuroblastoma, previously described by our group. The delineation of these two critical regions provided further evidence for a role of at least two tumor suppressor genes on 1p involved in MCC development. *TP73*, a *TP53* family member and located at 1p36.33 was investigated as a functional and positional candidate tumor suppressor in MCC. A missense mutation which could possibly reduce the transcriptional transactivation function of the amino-terminal region of *TP73* was detected in one MCC tumor. Our results showed that *TP73*, analogous to other cancers, is infrequently mutated in MCC. However, we can not exclude the possibility that other mechanisms could lead to inactivation of *TP73* or the fact that dominant negative splice variants can exert a role in MCC tumorigenesis. Future research should clarify these hypotheses. *TP53* mutation analysis, which was conducted in parallel, showed for the first time inactivating mutations in 19% of the investigated MCCs, indicating implication of *TP53* in the pathogenesis or progression of a subset of MCCs as observed for many other tumor types. The finding of typical UVB induced mutations in one MCC cell line provided further evidence for sun-exposure in the etiology of this skin tumor.

Whole genome screening methods including karyotyping, CGH and multiplex FISH were applied to search for previously unnoticed chromosomal imbalances in MCC. CGH led us to successfully identify a characteristic pattern of recurrent gains (1, 3q, 5p, 8q, 19 and X) and losses (3p, 5q, 8p, 10, 11q, 13q and 17p) pointing at chromosomal positions of candidate tumor suppressor genes or proto-oncogenes involved in MCC. Interestingly, the pattern of gains and losses resembled the CGH patterns previously reported for SCLC. These genetic similarities could point to common pathways or genes involved in the

development or progression of both MCC and SCLC. The new finding of recurrent loss of chromosome 10 in MCC was further explored by molecular analysis of the tumor suppressor gene, *PTEN*, located at 10q23. Despite the high frequency of LOH at the *PTEN* locus, only one homozygous deletion and one mutation, respectively were detected. The latter mutation was a nonsense mutation located in the phosphatase domain of *PTEN* and could result in a reduced or complete loss of the tumor suppressor function of *PTEN*. Our results suggested that loss of one *PTEN* allele and disruption of the second allele by mutation or homozygous deletion is a relatively rare event in MCC. We propose that other mechanisms such as *PTEN* haploinsufficieny or alternatively other tumor suppressor genes on chromosome 10 may be involved in MCC tumorigenesis.

The gene expression profiles of MCC and SCLC cell lines were determined by the use of Atlas cDNA expression arrays. We identified a set of 23 biological markers which allow improved differential diagnosis of MCC and SCLC. In addition, typical gene expression signatures associated with Variant and Classic MCC subtypes were identified for the first time. Further investigation of the gene defects and disrupted pathways involved in MCC and SCLC will lead to identification of molecular targets for new therapeutic interventions and reveal insight into the molecular basis of the heterogeneous biology of both aggressive tumors.

In conclusion, our work has provided new insights into the genetic mechanisms leading to MCC development and could direct future research hopefully leading to an improved survival of patients with MCC.

SAMENVATTING

Merkel cel carcinoom (MCC) is een zeldzame agressieve huidtumor van neuroendocriene oorsprong en wordt vooral gediagnosticeerd bij oudere personen. Deze aandoening komt hoofdzakelijk voor op die lichaamsdelen die aan zonlicht zijn blootgesteld. Dit suggereert dat UV stralen een rol zouden kunnen spelen in de ontwikkeling van MCC. Merkel cellen, de mechanoreceptoren van de huid, worden geassocieerd met het ontstaan van MCC. Bij de aanvang van deze thesis was slechts zeer weinig informatie beschikbaar omtrent de moleculaire mechanismen die zouden aanleiding kunnen geven tot MCC, en de reeds uitgevoerde cytogenetische studies waren zeer beperkt. Deze studies rapporteerden hoofdzakelijk 1p abnormaliteiten, vaak leidend tot verlies van 1p36, doch leverden zeer weinig of geen informatie omtrent de betrokkenheid van andere chromosomale regio's in MCC. Eén van onze initiële doelstellingen was dan ook het genereren van nieuwe informatie over genetische afwijkingen in MCC. Daartoe werden verschillende cytogenetische en moleculaire technieken aangewend. Daarnaast is het reeds gekend dat MCC klinische en immunohistochemische kenmerken deelt met kleincellig longcarcinoom (SCLC), eveneens een neuroendocriene tumor. Beide tumoren worden ook vaak differentieel gediagnosticeerd. Om een beter inzicht in de moleculaire biologie van beide tumoren te verwerven, werd besloten om de genexpressieprofielen van MCC en SCLC te bepalen en vervolgens de bekomen data in detail verder te bestuderen.

Op basis van het frequent voorkomen van 1p afwijkingen in MCC, besloten wij in eerste instantie deze regio te bestuderen. Dit resulteerde in de afbakening van twee subregio's betrokken in MCC. Als gevolg van een ongebalanceerde structurele herschikking constateerden wij verlies van het meest distale deel van 1p36 (1p36.31→pter) in een patiënt met MCC. Ook in diverse andere tumor types wordt een dergelijke deletie vaak teruggevonden. De distale 1p36 regio zou mogelijks een (geïmprint) tumorsuppressor-gen kunnen bevatten. Identificatie van een 1p36.2 insertiebreukpunt in de MCC cellijn UISO wees op de betrokkenheid van een tweede, meer proximaal gelegen regio in MCC. Dit breukpunt werd vervolgens gemapt binnen een groep van repetitieve genen en bleek samen te vallen met het 1p breukpunt van een constitutionele t(1;17) translocatie in een patiënt met neuroblastoom, welke werd gerapporteerd door onze onderzoeksgroep. De identificatie van deze twee kritische regio's levert bijkomend bewijs dat minstens twee tumorsuppressor-genen gelocaliseerd op 1p, een rol kunnen spelen in de ontwikkeling van MCC. *TP73*, een *TP53* homoloog, gelocaliseerd op 1p36.33 werd vervolgens als een functioneel en structureel kandidaat tumorsuppressor-gen onderzocht in MCC. Eén "missense" mutatie werd gedetecteerd in het amino-terminaal gedeelte van *TP73* en zou mogelijks tot een verstoring van de transcriptionele transactivatie functie van *TP73* kunnen leiden. Uit onze resultaten blijkt dat, analoog aan andere tumor types, mutaties in het *TP73* gen slechts zelden voorkomen. Ondanks deze lage *TP73* mutatie frequentie in MCC sluiten we niet uit dat andere mechanismen kunnen leiden tot *TP73* inactivatie of dat mogelijkse dominant negatieve isovormen een rol kunnen spelen in MCC tumorigenese. Verder onderzoek zou bovengenoemde hypothesen moeten bevestigen. In dezelfde studie werd ook het *TP53* gen onderzocht door middel van mutatie-analyse. Onze resultaten toonden voor de eerste maal inactiverende *TP53* mutaties aan in 19% van de onderzochte MCC's, wat ook wijst op een causale rol van het *TP53* gen in de pathogenese en progressie van een aantal MCCs. Het vinden van een typische UVB gerelateerde mutatie in één MCC cellijn onderstreept ook het belang van de invloed van zonlicht bij het ontstaan van deze huidtumor.

Methodes zoals karyotypering, CGH en M-FISH, waarbij het volledige genoom in één experiment bestudeerd wordt, werden aangewend om nog niet gekende structurele en numerieke chromosoomafwijkingen te identificeren in MCC. Vergelijkende genoomhybridisatie op een grote reeks MCC tumoren resulteerde in een typisch patroon van chromosomale toenames (1, 3q, 5p, 8q, 19 en X) en verliezen (3p, 5q, 8p, 10, 11q, 13q en 17p). Het vinden van dergelijke regio's is van groot belang omdat juist daar mogelijkse kandidaat tumorsuppressor-genen of proto-oncogenen kunnen liggen die een rol kunnen spelen in MCC. Het karakteristiek CGH patroon van MCC bleek tevens ook sterke gelijkenissen te vertonen met dat van reeds eerder beschreven CGH patronen van SCLCs. Deze genetische overeenkomsten zouden kunnen wijzen op een aantal gemeenschappelijke genen en signaaltransductiewegen betrokken in de ontwikkeling of progressie van zowel MCC als SCLC. Het feit dat chromosoom 10 herhaaldelijk gedeleteerd bleek te zijn in MCC, zette ons er toe aan om een moleculaire analyse van het tumorsuppressor-gen *PTEN*, gelocaliseerd op 10q23, aan te vatten. Ondanks de hoge incidentie van LOH ter hoogte van de *PTEN* locus, detecteerden we slechts één homozygote deletie en één mutatie. Deze laatste was een "nonsense" mutatie gelegen in het fosfatase domein van het *PTEN* gen en zou de tumorsuppressor functie van *PTEN* kunnen reduceren of zelfs volledig uitschakelen. Onze resultaten suggereren dat bij verlies van één *PTEN* allel zelden een mutatie in het tweede allel of een homozygote deletie optreedt bij MCC. Daarom stellen wij voorop dat andere mechanismen zoals *PTEN* haplo-insufficiëntie of eventueel andere al dan niet gekende tumorsuppressor-genen gelegen op chromosoom 10 betrokken kunnen zijn in de oncogenese van MCC.

"Atlas" cDNA expressie roosters werden aangewend om de genexpressieprofielen van MCC en SCLC cellijnen te bepalen. Via data-analyse werd een set van 23 biologische merkers bekomen die toelaten om de differentiële diagnose van MCC en SCLC te verbeteren. Tevens werden voor de eerste maal typische genexpressiepatronen voor "variante" en "klassieke" MCC subtypes geïdentificeerd. Verder onderzoek van genetische defecten en verstoorde moleculaire mechanismen in MCC en SCLC zal leiden tot identificatie van moleculaire doelwitten om mogelijkse nieuwe therapieën te ontwikkelen en zal zeker ons inzicht in de moleculaire basis en heterogeniteit van beide agressieve tumoren verhogen.

Samengevat kunnen we stellen dat ons werk zeker heeft bijgedragen tot het verkrijgen van nieuwe inzichten in de genetische mechanismen die optreden tijdens het ontstaan van MCC. Hopelijk zal toekomstig onderzoek leiden tot hogere overlevingskansen bij patiënten met MCC.

RÉSUMÉ

Le carcinome à cellules de Merkel (MCC) est une tumeur neuro-endocrine rare et aggressive qui se manifeste le plus souvent chez des patients agés. Il se déclare surtout sur des parties de peau exposées au soleil ce qui suggère une influence externe des rayons ultraviolets. Les carcinomes neuro-endocrines de la peau sont généralement considérés comme une prolifération maligne des cellules de Merkel. Celles-ci sont des mécanorécepteurs de la peau. Lorsque nous avons débuté ce travail, les mécanismes moléculaires à l'origine des MCC restaient inexplorés. Seules quelques données cytogénétiques étaient connues. En particulier, des anomalies récurrentes du bras court du chromosome 1 avec perte fréquente de la région 1p36 avaient été décrites. Le premier but de cette thèse était la recherche de nouvelles aberrations génétiques dans les carcinomes à cellules de Merkel au moyen de techniques (cyto)génétiques et moléculaires. En outre, des études faisaient mention de caractéristiques cliniques et immunohistochimiques similaires entre les MCC et les carcinomes à petites cellules du poumon (SCLC), une autre tumeur neuro-endocrine. De plus, un diagnostic différentiel entre les deux tumeurs doit souvent être posé. Afin de mieux comprendre la biologie moléculaire des MCC et des SCLC, nous avons étudié et comparé les profils d'expression géniques de ces deux tumeurs.

Des anomalies récurrentes de la région 1p36 étant décrites, nous avons dans un premier temps étudier en détail cette région chromosomique. Deux régions critiques distinctes ont été mises en évidence en 1p36 : une délétion de la région distale 1p36.31-pter suite à un réarrangement non balancé a été retrouvée dans une tumeur. Cette même région est également délétée dans d'autres types de tumeurs et est supposée contenir un gène suppresseur de tumeur. Une seconde région critique plus proximale avec point de cassure en 1p36.2 a également été mise en évidence dans la lignée UISO. Ce point de cassure (situé dans un cluster de gènes répétitifs) est identique à celui d'un patient avec neuroblastome décrit précédemment par notre groupe. L'observation de ces deux régions critiques prouve que deux gènes suppresseurs au moins doivent être impliqués dans les MCC. Le gène *TP73*, de la famille des *TP53* et localisé en 1p36.33 est un candidat potentiel. Une mutation faux-sens pouvant reduire la fonction transcriptionelle de la région amino-terminale du *TP73* a été observée dans un MCC. Cependant, comme dans d'autres cancers, nos résultats ont montré que *TP73* est rarement muté dans les MCC. Néanmoins, il n'est pas exclu que d'autres mécanismes mènant à l'inactivation de *TP73* ou à des épissages alternatifs (avec formation de variants dominants) soient impliqués dans le développement tumoral des MCC. L'élucidation de ces mécanismes demandent toutefois des recherches complémentaires. La recherche de mutation du *TP53* faite en parallelle, montre pour la première fois des mutations inactivantes dans 19% des MCC's étudiés et démontre l'implication de *TP53* dans la pathogénèse ou dans la progression d'un sous groupe de MCC comme cela a été montré dans plusieurs autres types tumoraux. L'observation de mutations induites dans une lignée de MCC après exposition aux UVB confirme l'influence de l'exposition solaire dans ce cancer de la peau.

Différentes méthodes telles que le caryotype, l'hybridation génomique comparative (CGH) et l'hybridation fluorescente multiplex (M-FISH) ont été utilisées afin de rechercher des aberrations chromosomiques de structure non balancées dans les MCC's. La CGH a permis la caractérisation de gains (1, 3q, 5p, 8q, 19 et X) et de pertes recurrents (3p, 5q, 8p, 10, 11q, 13q et 17p) déterminant ainsi des régions chromosomiques porteuses de gènes suppresseurs ou de protooncogènes potentiellement impliqués dans les MCC. Il est intéressant de constater que ce tableau est semblable à celui déterminé dans les SCLC. La

similitude des anomalies génétiques pourrait ainsi objectiver l'existence de mécanismes ou de gènes communs impliqués dan l'étiologie de ces 2 entités. La découverte de la perte fréquente du chromosome 10 dans les MCC nous a conduit à étudier le gène suppresseur *PTEN* (localisé en 10q23) par biologie moléculaire. Malgré le taux élevé de LOH dans le locus *PTEN*, nous avons seulement retrouvé une déletion homozygote et une mutation. Cette dernière est une mutation non-sens dans le domaine phosphatase du *PTEN*, ce qui pourrait réduire ou supprimer la fonction du gène *PTEN*. Les résultats démontrent que la perte d'une allèle *PTEN* et la perte d'activité par mutation du deuxième allèle ou la délétion homozygote sont des évenements rares dans les MCC's. D'autres mécanismes doivent être envisagés dans la génèse des MCC's tels que l'haplo-insuffisance ou l'implication d'autres gènes suppresseurs portés par le chromosome 10.

Les profils d'expression génique de 2 lignées dérivant d'un MCC et d'un SCLC ont été déterminés en utilissant "l'Atlas cDNA expression arrays". Ainsi, 23 marquers biologiques ont été isolés et identifiés qui permettent un diagnostic différentiel entre les MCC et les SCLC. De plus, des profils d'expression géniques distincts, associés de manière spécifique aux sous types de MCC "variantes" ou "classiques" ont été identifiés pour la première fois. Des études complémentaires des gènes défectueux et des mécanismes impliqués dans le développement des MCC's et des SCLC's autoriseront l'élaboration de nouveaux agents thérapeutiques dirigés spécifiquement contre de nouvelles cibles moléculaires et une meilleure compréhension de la base moleculaire et de la biologie heterogène de ces deux tumeurs aggressives.

En conclusion, notre travail a permis l'accumulation de données nouvelles sur les mécanismes génétiques impliqués dans le développement des MCC. Elles devraient permettre une meilleure approche thérapeutique et une amélioration de la survie des patients souffrant de MCC.

LIST OF ABBREVIATIONS

A12M2	adenovirus integration site
ABL	Abelson oncogene
AKT	protein-serine/threonine kinase AKT
APC	adenomatous polyposis coli
APUD	amine precursor uptake and decarboxylation
ATM	ataxia-telangiectasia mutated
BCC	basal cell carcinoma
BCR	breakpoint cluster region
BMM	bone marrow metastasis
CCD	charge coupled device
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary deoxyribonucleic acid
CIN	chromosomal instability
CGH	comparative genomic hybridization
CH A	chromogranin A
CK	cytokeratin antigen
CK20	cytokeratin 20
CL	cell line
CML	chronic myelogenous leukemia
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DEAC	diethylcoumarine
DNA	deoxyribonucleic acid
DMBT1	deleted in malignant brain tumor 1
DOP-PCR	degenerated oligonucleotide primer polymerase chain reaction
DPC4	deleted in pancreatic carcinoma 4
EMA	epithelial membrane antigen
ENO1	enolase 1
FITC	fluorescein isothiocyanate
FHIT	fragile histidine traid
GFAP	glial filament acidic protein
h-neu	human homolog of the Drosophila neutralized gene
Gy	Gray

HNPCC	hereditary nonpolyposis colorectal cancer
IGH	immunoglobulin heavy chain
JPS	juvenile polyposis syndrome
LCA	leucocyte common antigen
LGI1	leucine-rich, glioma inactivated 1
LNM	lymph node metastasis
LOH	loss of heterozygosity
M	metastatic tumor
MAPK	mitogen-activated protein kinase
MCC	Merkel cell carcinoma
M-FISH	multiplex fluorescence in situ hybridization
MIN	microsatellite instability
MLH1	mutL homolog 1
MSH2	mutS homolog 2
MXI1	max interactor 1
MYC	avian myelocytomatosis viral (v-myc) oncogene homolog
MYCL	avian myelocytomatosis viral (v-myc) oncogene homolog 1
MYCN	neuroblastoma derived avian myelocytomatosis viral (v-myc) related oncogene
NB	neuroblastoma
NF1	neurofibromatosis type 1
NFF	neonatal foreskin fibroblast
NFP	neurofilament protein
nm	nanometer
NSE	neuron-specific enolase
P	primary tumor
PI3K	phosphatidylinositol 3-kinase
PM	pulmonary metastasis
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTPase	protein tyrosine phosphatase
RASSF1	ras association domain family 1
RB1	retinoblastoma gene 1
REC	recurrence at site
RIZ1	retinoblastoma protein-interacting zinc finger 1
RNA	ribonucleic acid

RT-PCR	reverse transcriptase polymerase chain reaction
SAM	statistical analysis of microarrays
SCLC	small cell lung carcinoma
SCC	squamous cell carcinoma
SEMA3B	semaphorin 3B
SNP	single nucleotide polymorphism
SO	spectrumorange
SRO	shortest region of overlap
SSC	sodium saline citrate
SSCP	single strand conformation polymorphism
TMA	tissue microarrays
TP53	tumor protein p53 (Li-Fraumeni syndrome)
TP73	tumor protein p73
TR	texasred
TTF-1	thyroid transcription factor-1
UV	ultraviolet light
VIP	vasoactive intestinal peptide
VHL	von Hippel-Lindau
WCL	whole chromosome loss
WCG	whole chromosome gain

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J.H. Leonard, D. Nancarrow, N. Hayward, M. Van Gele, N. Van Roy, F. Speleman (2000): Deletion mapping of the short arm of chromosome 1 in Merkel cell carcinoma.
Cancer Detection and Prevention, 24: 620-627.

N. Van Roy, H. Van Limbergen, J. Vandesompele, M. Van Gele, B. Poppe, G. Laureys, A. De Paepe and F. Speleman (2000): Chromosome 2 short arm translocations revealed by M-FISH analysis of neuroblastoma cell line. Medical Pediatric Oncology, 35: 538-540.

M. Van Gele, J.H. Leonard, N. Van Roy, A.L. Cook, A. De Paepe, and F. Speleman (2001): Frequent allelic loss at 10q23 but low incidence of *PTEN* mutation in Merkel cell carcinoma. Int J Cancer, 92: 409-413.

G. Steele-Perkins , W. Fang , X.H. Yang , M. Van Gele, T. Carling , J. Gu, I.M. Buyse , J.A. Fletcher , J. Liu, R. Bronson , R.B. Chadwick, A. de la Chapelle, X. Zhang , F. Speleman and S. Huang (2001): Tumor formation and inactivation of RIZ1, an Rb-binding member of a nuclear protein-methyltransferase superfamily. Genes Dev 15: 2250-2262.

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INTERNATIONAL ORAL PRESENTATIONS

EMS/cortactin and *cyclin D1* gene amplification in neuroblastoma.
Meeting of the Belgian and Dutch Societes for Microscopy: "Information Beyond the Microscopical Image", December 12-13th, 1996, "Het Pand", Ghent, Belgium.

Identification of two distinct regions on 1p36 involved in Merkel cell carcinoma.
Seventh International Workshop on Chromosomes and Solid Tumors, January 20-22th, 1997, Tucson, Arizona, USA.

Physical and transcript map of the 17q-breakpoint of the constitutional 1;17 translocation found in a patient with neuroblastoma.
Advances in Neuroblastoma Research, June 15-17th, 1998, City of Bath, UK. "Young Investigator Award"

Mutation analysis of the *RIZ* gene and gene expression studies by "real-time" quantitative RT-PCR in neuroblastoma and Merkel cell carcinoma: an update.
The Burnham Institute, Cancer Research Center, 10901 North Torrey Pines Road, La Jolla, CA, USA, April 7th, 2000 (Guest speaker).

Molecular cytogenetic analysis of Merkel cell carcinoma: an overview.
Queensland Radium Institute Laboratory, Queensland Institute for Medical Research, Royal Brisbane Hospital Post Office, Brisbane 4029, Queensland, Australia. August 17th, 2000 (Guest speaker).

Comparative gene expression profiling of Merkel cell carcinoma and small cell lung carcinoma. 10th International Congress of Human Genetics, May 15-19th, 2001, Austria Center Vienna, Vienna, Austria.

CONGRESSES, WORKSHOPS AND TRAINING COURSES

III. Plenary workshop of the European Concerted Action on "Automation in molecular cytogenetic analyses". November 16-18th, 1995, Ghent, Belgium.

IV. Plenary workshop of the European Concerted Action on "Automation of molecular cytogenetic analyses". November 7-9th, 1996, Noordwijk, The Netherlands.

Meeting of the Belgian and Dutch Societes for Microscopy: "Information Beyond the Microscopical Image", December 12-13th, 1996, "Het Pand", Ghent, Belgium.

Seventh International Workshop on Chromosomes and Solid Tumors, January 20-22th, 1997, Tucson, Arizona, USA.

Symposium:"From the Gene to the Clinic", November 14-15th, 1997, Ghent, Belgium.

Bioinformatics training course in the Belgian EMBnet node, January, 6, 13 and 27th, 1998, VUB, Brussels, Belgium.

Advances in Neuroblastoma Research, June 15-17th, 1998, City of Bath, UK.

2nd Cold Spring Harbor Meeting on Cancer Genetics & Tumor Suppressor Genes, August 19-23th, 1998, CSHL, New York, USA.

Belgian Association for Cancer Research, January 30th, 1999, Antwerp, Belgium.

Genomics and Proteomics – Highway to Functional Biology, May 7th, 1999, Ghent, Belgium.

"Symposium on imprinting", October 15-16th, 1999, Brussels, Belgium.

GENETICS, NWO-MW retraite 'Rolduc', November 11-12th, 1999, Kerkrade, The Netherlands.

Introduction to PCR and Real Time PCR analysis, December 10th, 1999, Affligem, Belgium.

Belgian Association for Cancer Research, "Invasion and Metastasis", January 29th, 2000, Ghent, Belgium.

91st AACR Annual Meeting, April 1-5th, 2000, San Francisco, CA, USA.

377 Genetic Analyzer User Training, May 17-19th, 2000, The Netherlands.

Signal transduction in cell death and gene induction: From cells to physiology & strategies for discovery, November 10th and 11th, 2000, Ghent, Belgium.

Belgian Society of Human Genetics, First Annual Meeting, February 9th, 2001, Museé de la Médicine, Campus CHU Erasme, Brussels, Belgium.

The 2nd Belgian Bioinformatics Conference, April 6th, 2001, Ghent University, Ghent, Belgium.

10th International Congress of Human Genetics, May 15-19th, 2001, Austria Center Vienna, Vienna, Austria.

"CGH workshop", September 12th, 2001, Rotterdam, The Netherlands.

Course Bio-informatics 2001, September 20th, 26th and October 3rd, 2001, "Het Pand", Ghent, Belgium.

Enabling Discovery Seminar: Genotyping in the Genomic Era, February 27th, 2002, Engels Rotterdam, Rotterdam, The Netherlands.

3rd Belgian Bioinformatics Conference, April 12th, 2002, University of Namur, Namur, Belgium.

TRAININGS AT FOREIGN RESEARCH UNITS

Sanofi Recherche, Innopole B.P. 137, 31676 Labege CEDEX, France, February 22-28th, 1998. Training in sequence analysis of *TP73* and *TP53* (supervised by Dr. D. Caput and M. Kaghad).

Queensland Radium Institute Laboratory, Queensland Institute for Medical Research, Royal Brisbane Hospital Post Office, Brisbane 4029, Queensland, Australië, July-August, 2000. Training in Atlas cDNA expression array hybridizations combined with data analysis (supervised by Dr. H. Leonard and Dr. G. Boyle).